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DIABETIC GLOMERULOSCLEROSIS: ELECTRON AND LIGHT MICROSCOPIC STUDIES*

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Diabetic glomerulosclerosis is a frequent complication of diabetes mellitus of long duration, despite the efficacy of insulin therapy. Although the glomerular lesions have been studied extensively, the pathogenesis of this condition remains obscure. The present investigation was conducted in order to obtain further information on the evolution of diabetic glomerulosclerosis through the combined use of electron and light microscopic techniques on renal biopsies of diabetic patients. Recent studies by Brun and his associates,[1] Taft, Finckh and Joske,[2] and Darnaud, Denard, Moreau and Suc[3] have demonstrated the usefulness of renal biopsy for histologic examination of the glomerular lesions of diabetes. Previous reports from this laboratory[4-6] have shown that renal tissues obtained by biopsy provide excellent material for electron microscopy.

In diabetic glomerulosclerosis, the initial lesion observed by electron microscopy was a thickening of the basement membrane proper. The hyaline deposits of diabetic glomerulosclerosis were found to be extracellular, lying between adjacent endothelial cells rather than in the intercapillary space, as originally proposed by Kimmelstiel and Wilson.[7] The present studies also provided additional information on the relationship between the diffuse and nodular lesions, the nature and location of exudative lesions, and the differences between the glomerular lesions of diabetes and those seen in other conditions affecting glomeruli.

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Material and Methods

A group of 7 patients with diabetes mellitus were subjected to biopsy, using the needle biopsy technique of Lusted, Mortimore and Hopper.[8] The specimens were immediately cut into 4 to 6 segments. One half of the segments were fixed in formalin and the remainder in buffered osmium tetroxide.[9] The formalin-fixed tissue was embedded in paraffin and sectioned at 6 μ. The sections were stained with hematoxylin and eosin, the periodic acid-Schiff reaction (PAS), Masson's trichrome, phosphotungstic acid-hematoxylin (PTAH), a colloidal iron-cochineal technique,[10] or a combined colloidal iron and periodic acid-Schiff stain.[10] The PAS technique, colloidal iron-cochineal method, and the combined colloidal iron-PAS technique were the most useful in determining the basic glomerular alterations in paraffin-embedded tissues. In early lesions of diabetic glomerulosclerosis, there were deposits of PAS-positive material in the axial capillaries of the glomerulus. With the colloidal iron-cochineal technique, the deposits were orange to yellow.

The osmium tetroxide-fixed tissues were cut into 0.5 to 1 mm. cubes, dehydrated in graded alcohols and embedded in methacrylate. Ultrathin sections (less than 0.1 μ thick) were prepared for electron microscopy and examined and photographed according to methods described previously.[4,11]

Sections of osmium-fixed, methacrylate-embedded tissue were cut at 1 to 6 μ for light microscopy and were also stained with hematoxylin, the periodic acid-Schiff stain, a fuchsin-alum-hematoxylin method (FAH),[12] Masson's trichrome, the Mallory-Heidenhain azan, or a gallocyanin-chromium-phloxine method.[13] * Osmium fixation gave excellent preservation, and in thin methacrylate sections, 1 to 4 μ, finer details could be observed by light microscopy than in formalin-fixed, paraffin-embedded tissues. Moreover, staining of osmium methacrylate tissues provided a means of studying the same glomerulus by light and electron microscopy. In sections stained with hematoxylin, the basement membrane and deposits of hyalin were unstained and had a glassy, refractile appearance. The PAS technique gave excellent results with both methacrylate and paraffin-embedded tissues. The gallocyanin-chromium-phloxine method on methacrylate-embedded tissues produced a result similar to that observed in hematoxylin and eosin stained preparations of paraffin-embedded tissues.[13] The fuchsin-

* Tissues fixed in osmium and embedded in methacrylate did not react in the usual manner with many stains[11,14]; however, modifications of a number of staining methods were developed for methacrylate-embedded tissues.[12]

alum-hematoxylin method for staining of plastic embedded tissues[12] was found to be particularly useful for demonstrating the progressive alterations in the glomeruli of patients with diabetes. In these preparations the basement membrane, hyalin and endothelial cytoplasm were stained by basic fuchsin. In early lesions the hyalin was stained pink whereas in far advanced lesions it was deep purple (Figs. 1 to 4).

Observations

Clinical and Laboratory Data

Pertinent clinical and laboratory data in the group of 7 diabetic patients (21 to 49 years of age; 6 females, and 1 male), are summarized in Tables I and II. The diabetes mellitus was of relatively long duration in most patients, with onset in childhood in several;

Table I

Diabetic Glomerulosclerosis: Clinical Data

Case	Sex	Age	Duration of diabetes (yrs.)	Insulin (units per day)	Retinopathy*	Blood pressure	Edema
1	F	24	6	70	++	124/80	o
2	F	49	25	10 to 30	o	120/80	+
3	F	43	10	38	++	125/80	o
4	F	36	29	35	++	120/80	o
5	M	21	17	60	++	140/90	o
6	F	29	19	52	++++	180/100	+
7	F	43	18	10	+++	146/90	++

* Degree of severity: o to ++++.

Table II

Diabetic Glomerulosclerosis: Laboratory Data

Case	Serum cholesterol* (mg. per 100 ml.)	PSP excretion (% in 15 min.)	Creatinine clearance† (ml. per min.)	Proteinuria‡
1			69	o
2	200	35	70	o
3	250 to 340	29	110	0.4
4		35	94	o to ++
5		26	100	1.8
6	300	35	41	2.9
7	520	5	36	5.9

* Modified Sperry-Schoenheimer method.

† Corrected to body surface area of 1.73.

‡ 20 per cent sulfosalicylic acid for qualitative determinations; modified biuret method for quantitative determinations.

Degree of severity: o to ++++.

none required large doses of insulin. Two patients, cases 6 and 7, had the classical Kimmelstiel-Wilson syndrome, i.e., severe proteinuria, hypo-albuminemia, azotemia, edema, hypertension, and retinitis. The remaining patients had renal function within normal limits and were normotensive. Retinopathy was present in 6 patients. Proteinuria was present in 5 of the 7 individuals, and it was moderately severe in 3. Renal biopsy specimens were procured during hospitalization for a minimum period of 24 hours.

Observations by Light Microscopy

The degree of glomerular damage was evaluated in paraffin and methacrylate-embedded sections stained with PAS. As summarized in Table III, there was a wide range in the degree of renal abnormality as evaluated by light microscopy. In case 1, most of the glomeruli were normal or showed only minimal diffuse lesions. On the other hand, in case 7, all glomeruli were severely damaged and 5 were completely hyalinized. Cases 2 to 6 showed glomerular involvement intermediate between the two extremes. Diffuse glomerular alterations were seen in every patient, but only patients with more advanced renal involvement showed nodular or exudative glomerular lesions (cases 6 and 7). The focal nature of diabetic glomerulosclerosis was exemplified by the lesions in case 1. Of the 14 glomeruli in the specimen from this patient, 2 were completely hyalinized, 6 showed mild diffuse lesions with basement membrane thickening, and the remainder were essentially normal. The specimens from cases 2 and 3 also contained some glomeruli which appeared normal by light microscopy. However, in the remaining patients (cases 4 to 7), all of the glomeruli showed some abnormality.

The earliest lesions occurred in the primary bifurcations of the afferent arterioles. In methacrylate sections stained with FAH, these were visible as an increase in the fuchsinophilic material (hyalin) in the medial branches of the afferent arterioles; the peripheral loops were unaffected (Fig. 1). With greater glomerular damage, deposits appeared in the peripheral loops of glomeruli (Fig. 2). In severely damaged glomeruli, the central capillaries were obliterated by hyalin, and only a few peripheral loops remained patent (Fig. 3). Finally, many glomeruli were completely obliterated by such hyaline deposits (Fig. 4). In glomeruli with moderate damage, i.e., with stenosis of the central capillaries, the peripheral loops in apposition to Bowman's capsule were dilated and less tortuous than normal. These features were similar to those noted by Kimmelstiel and Wilson[7] who observed

that capillaries were frequently dilated at the periphery of nodules. In far advanced sclerotic lesions stained with hematoxylin, it was noted that the hyaline material had lost some of its glassy, refractile qualities, and in FAH preparations the deposits no longer stained pink but were purple or blue.

Exudative lesions of the glomerular capillaries were seen only in the patients exhibiting the most severe renal damage (cases 6 and 7). The "fibrinoid" constituting the exudative lesions was commonly located in the peripheral capillary loops (Fig. 6). Exudative lesions were readily recognized in PAS preparations because of their homogeneous appearance (Figs. 5 and 6). In sections stained with Masson's trichrome they were stained by ponceau-fuchsin in contrast to the diffuse and nodular lesions which were stained by light green.

TABLE III

Diabetic Glomerulosclerosis: Pathologic Lesions

Case	Glomeruli examined		Diffuse glomerular alterations*	Nodular glomerular alterations*	Capsular thickening*	Completely hyalinized glomeruli	Exudative lesions*
	Total no.	No. by electron microscope					
1	14	9	±	o	o	2	o
2	17	9	+	o	o	o	o
3	13	6	++	o	+	o	o
4	21	10	+++	+	++	o	+
5	23	10	+++	+	++	o	o
6	11	6	+++	+++	++	2	++
7	22	10	++++	+++	+++	5	+++

* Degree of severity: o to ++++.

Observations by Electron Microscopy

Normal Glomeruli. The fine structure of the normal glomerulus has been reviewed elsewhere.[4] As previously reported, there were only 3 components of the glomerulus: endothelium, basement membrane proper, and epithelium (Fig. 9). There was no evidence of an intercapillary tissue or mesangium. In some instances certain cells had the appearance of a "mesangium." However, by serial sectioning it was established that these represented either the base of endothelial cells near the point of their attachment to the basement membrane proper, or sections through smooth muscle cells in the wall of the afferent arteriole at the hilus.

The basement membrane proper has been shown to be thicker in human glomeruli than in those of laboratory animals.[4,15] Vernier[16]

measured glomerular basement membranes from 25 normal individuals, aged 11 hours to 84 years, and found those of older children and adults to have an average thickness of 2,700 to 3,000 Å from the base of the foot processes to the endothelial cell membrane. In infants and very young children, i.e., under 3 years of age, it was less than 1,200 Å. In the present investigation basement membranes averaging 3,000 Å or less were considered to be within normal limits.

Thickening of the Basement Membrane. Electron micrographs provided a more accurate method of determining the degree of thickening of the basement membrane proper than light microscopy. The "basement membrane," distinguished by light microscopy in hematoxylin and eosin stained preparations, included not only the basement membrane proper, but also the peripheral endothelial cytoplasm and epithelial foot processes.[4,6,17] In PAS or FAH preparations, both the basement membrane and the endothelial cytoplasm stained similarly.

A thickening of the basement membrane was seen in all glomeruli in patients with diabetes (Figs. 10 to 17). In cases 1, 2, and 3, some glomeruli had appeared normal by light microscopy. However, examination by electron microscopy revealed thickening of the basement membrane proper in these glomeruli. The degree of thickening varied, not only from one glomerulus to another but also from one loop to another in the same glomerulus. In case 1, all loops averaged at least 2 to 3 times normal thickness (Figs. 11 and 12), and some were 5 to 7 times normal thickness. In case 2, some loops were near the upper limits of normal whereas others were 2 to 5 times normal thickness. In cases 4 to 7, all of the basement membranes were thickened and measured 2 to 10 times normal (Figs. 13 to 17).

In a few instances the course of the basement membrane was very tortuous in contrast to its usual smooth contour. McManus[18] has pointed out that this wrinkling of the basement membrane probably occurred as a result of focal ischemic changes.

Diffuse Glomerular Lesions. In glomeruli containing minimal lesions, the hyaline deposits were similar in appearance to the material constituting the basement membrane proper (Figs. 10 to 13); in many instances the deposits were continuous with the basement membrane. Gradual obliteration of the capillary loops occurred by the enlargement of the hyaline masses. The deposits were located between the cell membranes of adjacent endothelial cells and were clearly extracytoplasmic; the endothelial cell membranes demarcated the endothelial cytoplasm from the hyaline mass.

In sections of early lesions, the relationship of a particular endothelial cell to the capillary lumen was not always apparent; how-

ever, with serial sectioning, the identity of the cell could be established by its location in the lumen of the capillary. With increased deposition of hyalin resulting in partial or complete obliteration of the capillary lumen, some of the cells became embedded in the hyaline mass and were isolated from the capillary lumen. The deposition of the hyalin was also accompanied by a slight increase in the number of endothelial nuclei.

Nodular Lesions. The only feature which distinguished the nodular lesion from the diffuse lesion was the size of the hyaline mass. In the diffuse process, the hyaline material was deposited in a uniform manner at the borders of endothelial cells, eventually resulting in the isolation of islands of cytoplasm in the expanding hyaline masses (Figs. 10 to 13). On the other hand, in the nodular lesion the hyaline material was deposited as a large mass crowding the endothelial cells to the periphery, and occasional individual cells were entrapped within the hyaline mass (Fig. 16). The nodular alterations were seen with increasing frequency in patients with more severe renal damage and were always associated with the diffuse changes.

In patients with severe renal damage, the hyaline deposits in glomeruli appeared heterogeneous and were fibrillary or granular in some areas (Figs. 14, 16 and 17). This was in sharp contrast to their homogeneous appearance in the early lesions.

Exudative Lesions. In electron micrographs the accumulations of fibrinoid which constituted the exudative lesions were characterized by their great density (Figs. 15 and 17). The material was clearly extracellular; it was found between the endothelial cell membrane and the basement membrane proper, i.e., subendothelial in position. The smallest deposits, presumably representing the earliest lesions, were of such a size that they could not be readily identified by light microscopy (Fig. 15). Larger masses bulged conspicuously from the capillary wall, filling and occluding the capillary lumens and forming "fibrin caps" (Fig. 17). With occlusion of the capillary lumen, the masses appeared to undergo degenerative alterations; the density of the deposits diminished and vacuoles appeared. The latter may have been due to fat[19] which had been lost in the preparation of the material (Fig. 19).

The exudative lesions of Bowman's capsule were subepithelial in position, lying between the parietal epithelium and the capsular basement membrane. The basement membranes of the proximal and distal convoluted tubules were frequently thickened (Fig. 18) and sometimes contained deposits of fibrinoid.

The early deposits of dense, fibrinoid substance were indistinguish-

able in position and appearance from the fibrinoid deposits seen in glomeruli in patients with disseminated lupus erythematosus (Figs. 7, 8, 21 and 22), glomerulonephritis and eclampsia (Fig. 24).

Visceral Epithelial Cells. In glomeruli with minimal or moderately advanced diffuse lesions, no abnormalities were present in the epithelium. It was particularly noteworthy that the organization of the epithelial cytoplasm into foot processes was normal (Figs. 10 to 12).

In severely damaged glomeruli the axial capillaries were obliterated and the urinary space narrowed by the expanding masses of hyalin. There was some coalescence of the epithelial foot processes in the areas showing pronounced hyaline accumulation, whereas the epithelial cells in contact with open loops retained their normal structure.

With further glomerular damage and obliteration of the peripheral loops, there was a coalescence of the foot processes, resulting in a spreading of the epithelial cytoplasm along the basement membrane (Figs. 16, 17 and 19). Hence, the alterations in the foot processes were most severe in the patients with the greatest glomerular damage (cases 6 and 7). Likewise, these patients were the only ones with clinical features associated with the nephrotic syndrome.

Arteriolar Lesions. In patients with far advanced glomerulosclerosis, there were deposits of abnormal substance or "hyalin" in afferent arterioles. The arteriolar hyaline deposits had a finely particulate texture and an appearance similar to fibrinoid. The deposit was observed beneath the internal elastic membrane and partially replaced the smooth muscle of the media (Fig. 20).

Diabetic Glomerulosclerosis Compared with Other Glomerular Lesions

It was shown in previous investigations that early in the course of nephrosis, glomerulonephritis, and disseminated lupus erythematosus, distinctive alterations occurred in the structure of the glomerulus when viewed by electron microscopy, which permitted the differentiation of one disease process from another. In this investigation the glomerular alterations of diabetes mellitus were compared with the glomerular lesions of disseminated lupus erythematosus, amyloidosis, pre-eclampsia and eclampsia.

Disseminated Lupus Erythematosus. The glomerular lesions of diabetic patients were compared with those in 6 patients with disseminated lupus erythematosus.[4,20,21] The basement membrane proper was thickened in both groups. Electron micrographs of glomeruli in lupus erythematosus revealed variable amounts of dense "fibrinoid" in all cases even though in 3 no typical "wire loops" were demonstrable by

light microscopy. The "fibrinoid" was usually located between the basement membrane and endothelial cytoplasm (Figs. 21 and 22). Occasionally it also apparently permeated the basement membrane and accumulated in patches between the basement membrane and epithelium (Fig. 21).

In patients with diabetic glomerulosclerosis, deposits of "fibrinoid" (i.e., exudative lesions) also occurred, but only in the severe forms; the pattern of deposition was quite different, for it was typically seen in the peripheral glomerular loops as "fibrin caps." Proliferation of endothelial cells occurred frequently in lupus, but not in diabetes mellitus. Loss of the epithelial foot processes occurred occasionally as a later manifestation in both diabetes mellitus and lupus erythematosus. Large hyaline deposits, similar in density to the basement membrane, were not found in lupus.

Nephrosis. A characteristic glomerular lesion found in all patients with the nephrotic syndrome was previously reported.[4-6] The lesion consisted of a loss of the normal delicate structure of the epithelial foot processes; the cytoplasm was spread along the basement membrane and exhibited irregularly spaced interruptions. This abnormality occurred early in lipoid nephrosis coinciding with the onset of proteinuria, and the glomeruli showed a more or less uniform degree of involvement. In diabetic glomerulosclerosis the distortion of the epithelial foot processes occurred as a late manifestation. This lesion was observed only in glomeruli with large hyaline and fibrinoid deposits (Figs. 17 and 19), and only in patients having clinical evidence of the Kimmelstiel-Wilson syndrome. Thus, in diabetes mellitus as well as in lipoid nephrosis,[20,21] there was a close correlation between massive proteinuria and the occurrence of the lesion of the epithelial foot processes.

Amyloidosis. The glomerular lesions of 3 patients with primary amyloidosis[21]* differed from those of diabetic glomerulosclerosis and were similar to those reported by Miller and Bohle[22,23] in mice with experimental amyloidosis. In amyloidosis there was irregular thickening of the basement membrane proper, with bulging of the thickened segment toward the epithelium (Fig. 23). The thickening was focal so that one segment measured 20 or more times normal, whereas an adjacent segment appeared quite normal. In the thickened regions, the basement membrane was lighter in density than normal and had a foamy appearance. These features permitted ready differentiation from the hyaline and "fibrinoid" deposits of diabetes mellitus.

* Diagnosis confirmed at necropsy.

Pre-eclampsia and Eclampsia. The lesions of diabetic glomerulosclerosis could be readily differentiated from those of pre-eclampsia and eclampsia.[21] Pronounced swelling of the endothelium and fibrinoid deposits in glomeruli were observed in patients with the latter conditions (Fig. 24). In many instances the endothelial cytoplasm on one side of a glomerular capillary was in contact with that on the opposite side, resulting in occlusion of the lumen. There were subendothelial and occasionally inter-endothelial deposits of "fibrinoid" in many capillaries. The mitochondria of the epithelial cells sometimes showed swelling, and there was "hyaline droplet" formation. The basement membrane was normal in thickness.

Discussion

It was noteworthy that alterations in glomerular structures were not necessarily correlated with the severity of diabetes as judged by insulin requirement, occurrence of diabetic coma, altered renal function, or proteinuria. On the other hand, the presence of retinopathy resulting from vascular disease coincided with the existence of renal lesions except in case 2. The severity of the retinopathy tended to parallel the severity of the renal alterations and the degree of hypertension. In all patients except one (case 1), diabetes mellitus had been present for periods ranging from 10 to 29 years. However, only two of the patients (cases 6 and 7) had far advanced glomerular lesions (+++ to ++++). One patient (case 2) had diabetes for 25 years, but had normal blood pressure, renal function, and eyegrounds.

Diabetic glomerulosclerosis, as observed by light microscopy, consisted of a progressive deposition of a hyaline substance initially observed near the primary branches of the afferent arterioles. Subsequently the more peripheral capillary loops became affected; eventually complete obliteration of glomeruli occurred.

One of the earliest lesions observed by electron microscopy in the glomeruli of patients with diabetes was a thickening of the basement membrane proper. Hyalin was very similar in appearance to the basement membrane and was sometimes in direct continuity with it. The hyalin lay between the cell membranes of adjacent endothelial cells, i.e., inter-endothelial, rather than "intercapillary" in position.[7,24] Irvine, Rinehart, Mortimore and Hopper[25] and Bergstrand and Bucht[26] have observed a thickening of the basement membrane and noted the close association between hyalin and endothelium in glomeruli in patients with diabetes. In contrast to our observations, however, they believed hyalin to be present within the endothelial cytoplasm.

No evidence for the existence of intercapillary tissue or "mesangium"

was noted in these investigations. This is in keeping with the observations of other investigators[25,26] of diabetic glomerulosclerosis by electron microscopy. Hall and Roth[27] and Kurtz[28] found that in embryonic rat and human glomeruli, glomerular capillaries develop *in situ* from unorganized masses of cells, i.e., the endothelial anlage. Thus, their observations cast doubt on the embryologic basis for a "mesangium."

The diffuse lesions of diabetic glomerulosclerosis were considered by Bell[29,30] to be as characteristic of diabetes mellitus as the nodular lesions if glomerulonephritis could be excluded. On the other hand, the majority of investigators[31] have considered the hyaline nodule to be the only glomerular lesion virtually pathognomonic of diabetes mellitus. Recent studies of renal biopsies by other investigators[1-3] as well as our own indicate that the nodular lesion is derived from the diffuse lesion, as originally proposed by Bell. In the present investigation, the hyaline nodule differed from the diffuse lesion only in size and shape. The stage at which the diffuse lesion was designated a "nodule" was necessarily arbitrary. The size of the hyaline mass was larger in the thicker sections because of the superimposition of structures. In sections measuring 0.5 to 2 μ in thickness, examined by light microscopy, this effect was greatly minimized and it was virtually eliminated in electron micrographs of ultrathin sections (less than 0.1 μ).

During recent years considerable attention has been directed to the exudative features of diabetic glomerulosclerosis. Although at times exudative lesions may be confused with the nodular lesions of Kimmelstiel and Wilson, the two types are actually quite distinctive in texture, tinctorial reaction and location.[32] Exudative lesions have been shown[33-36] to consist of deposits of fibrinoid[36,37] in the glomerular loops, Bowman's capsule, or in the basement membranes of the proximal convoluted tubules. The typical glomerular lesions were found in the peripheral loops adjacent to Bowman's capsule, in the form of crescentic, homogeneous masses, i.e., "fibrin caps." Interpretations of the exact significance and location of these lesions have varied considerably.[31,33,35]

In the present study the great density of fibrinoid in electron micrographs readily permitted its differentiation from the hyaline deposits of the diffuse and nodular lesions. In electron micrographs fibrinoid was demonstrated to be subendothelial in position, i.e., located between the endothelial cytoplasm and the basement membrane. Others have pointed out that exudative lesions were not specific for diabetes.[30,32,34] We have observed fibrinoid material identical in staining affinities, location and electron density in patients with subacute and

chronic glomerulonephritis, disseminated lupus erythematosus, and in eclampsia. Therefore, it seems likely that "fibrinoid" deposition is a nonspecific manifestation of severe glomerular damage. However, the gross pattern of "fibrinoid" deposition in diabetic glomerulosclerosis differs from that in other glomerular lesions; for the larger peripheral masses of fibrinoid or "fibrin caps" are more frequent in diabetes.

It has been shown that the hyaline deposit of diabetic glomerulosclerosis is acidophilic, argyrophilic,[37] and stains with PAS.[24] It has also been established that hyalin has certain distinctive features by phase microscopy[38] and ultraviolet absorption.[39] Our investigations by electron microscopy demonstrated that the early deposits of hyalin were similar in appearance to the basement membrane proper and were frequently continuous with this layer. In the more severe lesions, hyalin had a heterogeneous texture with fibrillary or vacuolated areas resembling the hyaline deposits of chronic glomerulonephritis.[4,5] The altered structural and tinctorial character of the hyalin in more advanced lesions may be due to a superimposition of changes resulting from severe arteriolosclerosis.

There have been various concepts concerning the derivation of hyalin, i.e., from connective tissue cells,[7] by splitting of basement membranes,[29,37] or by deposition from the circulating blood.[24] Studies of glomerular endothelium are pertinent to this problem. Investigations by Rinehart, Farquhar, Jung and Abul-Haj,[40] and Hall and his coworkers[17,27,41,42] have indicated that the endothelium forms and maintains the basement membrane. Our observations suggest that hyalin may, likewise, be derived from the endothelium.

In view of the direct continuity of the internal elastic membrane of the afferent arteriole with the glomerular basement membrane,[42] it would seem likely that both the arteriolar and glomerular abnormalities were the result of a common metabolic defect of the endothelium.

Summary

Renal biopsy specimens from 7 patients with diabetes mellitus were examined by light and electron microscopy. Clinically, the patients presented great variation in severity of renal impairment, ranging from 3 patients with normal renal function and blood pressure, to 2 patients with the classical features of the Kimmelstiel-Wilson syndrome (massive proteinuria, retinitis, hypertension, edema, azotemia and hypoalbuminemia).

By light microscopy diabetic glomerulosclerosis was characterized by gradual obliteration of glomeruli by the deposition of hyalin. Hya-

line deposit was evident initially near the afferent arteriole and subsequently extended radially to involve more peripheral loops.

By electron microscopy thickening of the basement membrane proper was one of the earliest manifestations of the disorder; some degree of thickening, up to 10 times normal, was observed in all glomeruli. The earlier lesions, i.e., the smaller deposits of hyalin, were similar in appearance to the basement membrane and were frequently continuous with it. Their presence between the cell membranes of adjacent endothelial cells (inter-endothelial) suggested that they represented an extracellular product of the glomerular endothelium. With greater accumulation of hyalin, islands of endothelial cytoplasm appeared isolated by the expanding masses. Finally, with further progression, the capillary lumen was obliterated. In the later stages, hyalin showed altered staining characteristics and was more heterogeneous in texture by electron microscopy. The nodular lesions developed from the diffuse lesions by enlargement of the hyaline deposits, as originally suggested by Bell.

Fibrinoid deposits or "exudative lesions" of glomeruli were present in two patients with the most advanced glomerular alterations. This substance could be distinguished in electron micrographs by its great density. It was subendothelial in position, lying between the endothelial cell membrane and the basement membrane.

The epithelium was not altered early in the course of hyaline deposition. Later, however, some loss of the epithelial foot processes was seen in severely damaged glomeruli.

The fine structural alterations of glomeruli in diabetes mellitus could be differentiated from those occurring in disseminated lupus erythematosus, nephrosis, amyloidosis, and pre-eclampsia and eclampsia.

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We are greatly indebted to Mrs. Karin Taylor, Miss Barbara Jennings, and Mr. Robert Brooks for their valuable technical assistance.

[*Illustrations follow*]

LEGENDS FOR FIGURES

Figures 1 through 8 are photomicrographs taken from renal biopsy tissues fixed in 1 per cent osmium tetroxide, embedded in n-butyl methacrylate, cut at 1 to 6 μ with a Porter-Blum microtome and stained as indicated. Figures 9 through 24 are electron micrographs taken from ultrathin sections (less than 0.1 μ in thickness) prepared from the same tissues. In many instances the electron micrographs are taken from sections contiguous with the thicker sections prepared for light microscopy.

Figures 1 to 4 illustrate glomeruli stained by the fuchsin-alum-hematoxylin (FAH) method, and show progressive glomerular damage in diabetic glomerulosclerosis. Increasing glomerular involvement is manifested by an increase in the amount of fuchsinophilic (pink to purple staining) material present. The endothelial cytoplasm and basement membrane proper, as well as the abnormal hyaline deposits, are demonstrated by FAH.

FIG. 1. Glomerulus from case 1 with minimal diffuse alterations which were not recognizable in an adjacent section stained with periodic acid-Schiff (PAS) stain. 2 μ section. × 250.

FIG. 2. Glomerulus from case 2, with more obvious diffuse alterations which are most severe in the central portion of the glomerulus, but also radiate out to affect more peripheral capillary loops. 5 μ section. × 200.

FIG. 3. Moderately severe diffuse alterations showing obliteration of the central portions of the glomerular lobules by the deposition of hyalin, from case 4. The number of patent lumens is greatly reduced. 2 μ section. × 250.

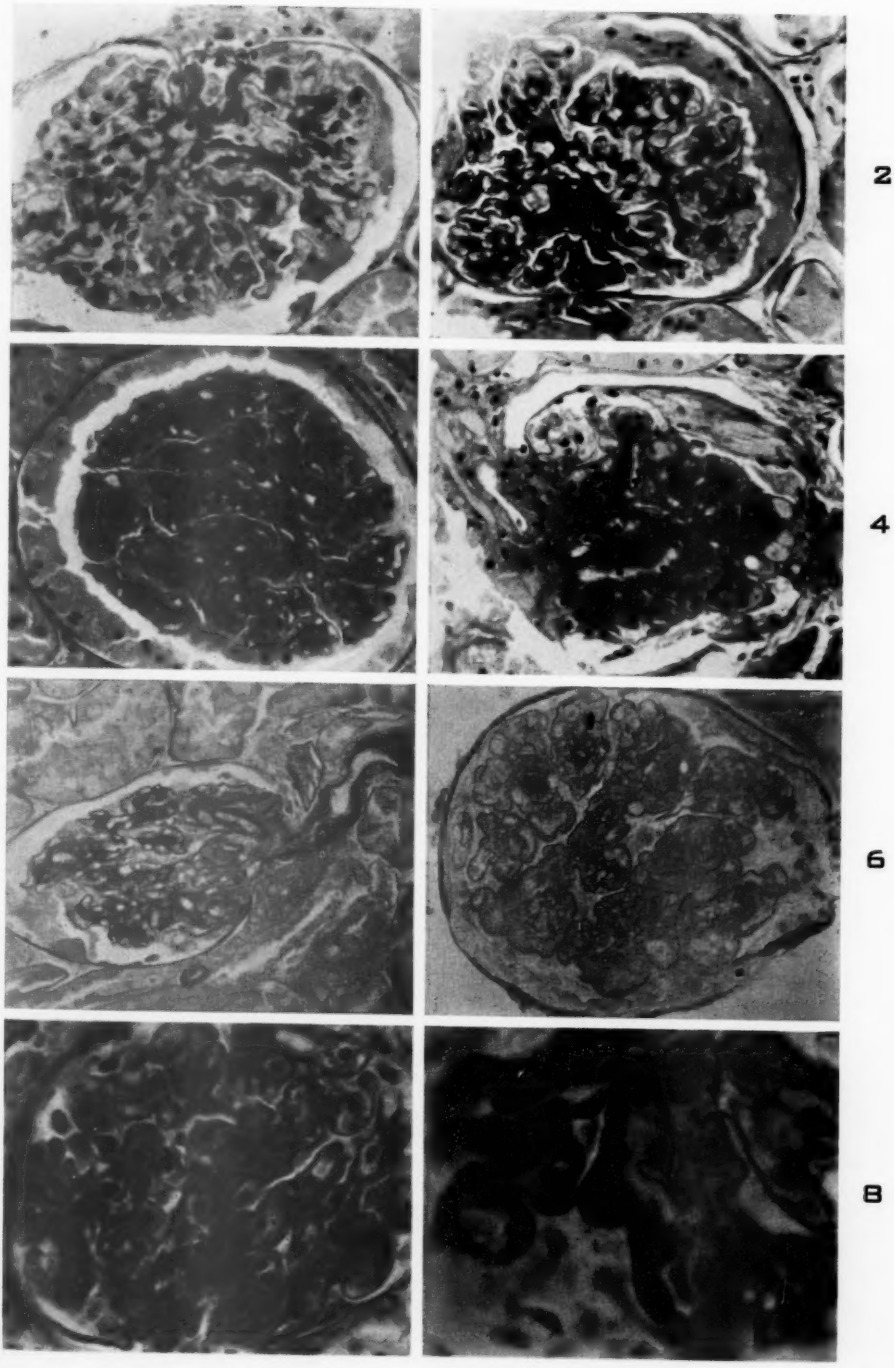
FIG. 4. Nearly total replacement of a glomerulus by hyalin, case 7. Only a few peripheral loops facing Bowman's capsule remain open. 2 μ section. × 200.

FIG. 5. Glomerulus from case 4, showing moderate diffuse alterations. Severe arteriolosclerosis is evident in the afferent arteriole. An exudative lesion is seen in the basement membrane of Bowman's capsule to the left. 4 μ section, PAS stain. × 180.

FIG. 6. Glomerulus from case 7, showing complete obliteration by hyaline deposits. In several peripheral capillary loops facing Bowman's capsule, exudative lesions or "fibrin caps" can be seen. These lesions may be distinguished from deposition of hyalin by their more intense reaction with PAS and their homogeneous appearance. 2 μ section, PAS stain. × 250.

FIG. 7. Disseminated lupus erythematosus; glomerulus demonstrating "fibrinoid." In this patient, deposits of "fibrinoid" could be seen in nearly every loop of each glomerulus. 6 μ section, Masson's trichrome stain. × 400.

FIG. 8. Disseminated lupus erythematosus; portion of another glomerulus, illustrating several "wire-loop" lesions. A "wire-loop" is composed of a layer of orange-red staining "fibrinoid" located beneath the basement membrane which is stained blue. 4 μ section, Mallory-Heidenhain azan stain. × 1200.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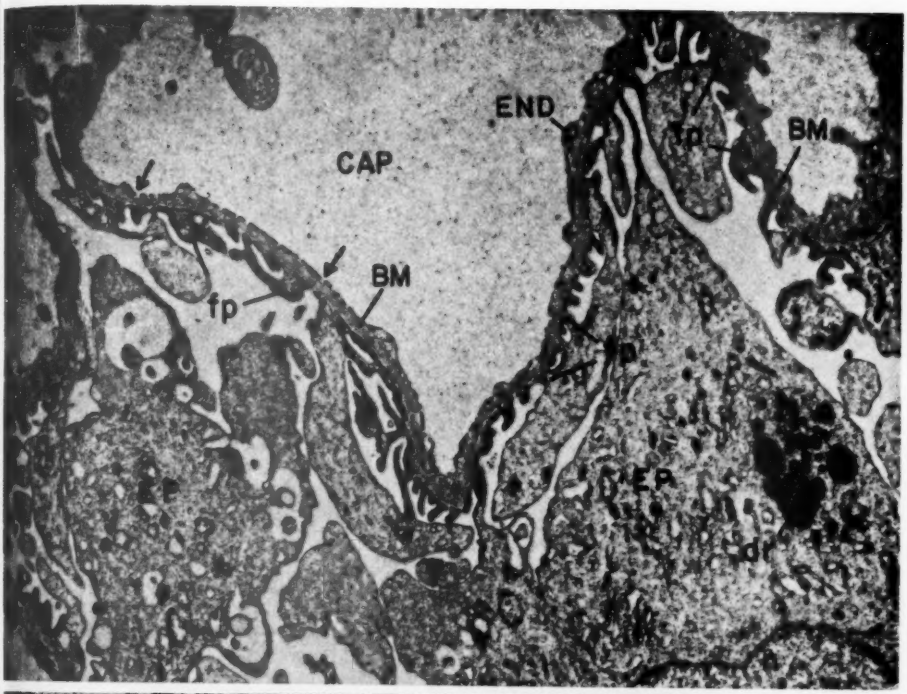
Key for Figures 9 to 24:

BM = basement membrane
END = endothelium
EP = epithelium
CAP = capillary lumen
H = hyalin
A = amyloid
RBC = red blood cell
BC = Bowman's capsule
SM = smooth muscle cell
EL = elastic membrane
fp = foot process
cm = cell membrane
fib = fibrinoid
dr = droplets
n = nucleus
m = mitochondria
vac = vacuoles

FIG. 9. Portion of a normal glomerulus from a 42-year-old non-diabetic man, showing the thickness of a normal basement membrane for comparison with succeeding figures from patients with diabetes. The basement membrane is a relatively homogeneous layer, averaging 2,600 Å in thickness from the base of the foot processes to the endothelial cell membrane. Between the basement membrane and the capillary lumen is a thin peripheral layer of endothelial cytoplasm, showing characteristic interruptions (arrows). No endothelial nuclei are present in the field. The cytoplasm of several epithelial cells and a segment of one epithelial nucleus is shown. The elaborate organization of the cytoplasm into numerous foot processes in contact with the outer surface of the basement membrane is visible. An aggregation of dense, ovoid droplets is present in the cytoplasm of the epithelial cell to the right. × 7,200.

FIG. 10. Portion of a glomerulus from case 2. By light microscopy, no abnormalities were detected; by electron microscopy the basement membrane measured approximately 5,500 Å, or twice normal thickness. Several small accumulations of hyalin are also present. This early or minimal hyaline deposit is very similar in appearance to normal basement membrane. In many instances it is continuous with the basement membrane, as shown here. The endothelial cell membrane may be seen between the endothelial cytoplasm and the hyalin. The epithelial cells and their foot processes appear unaltered. × 7200.



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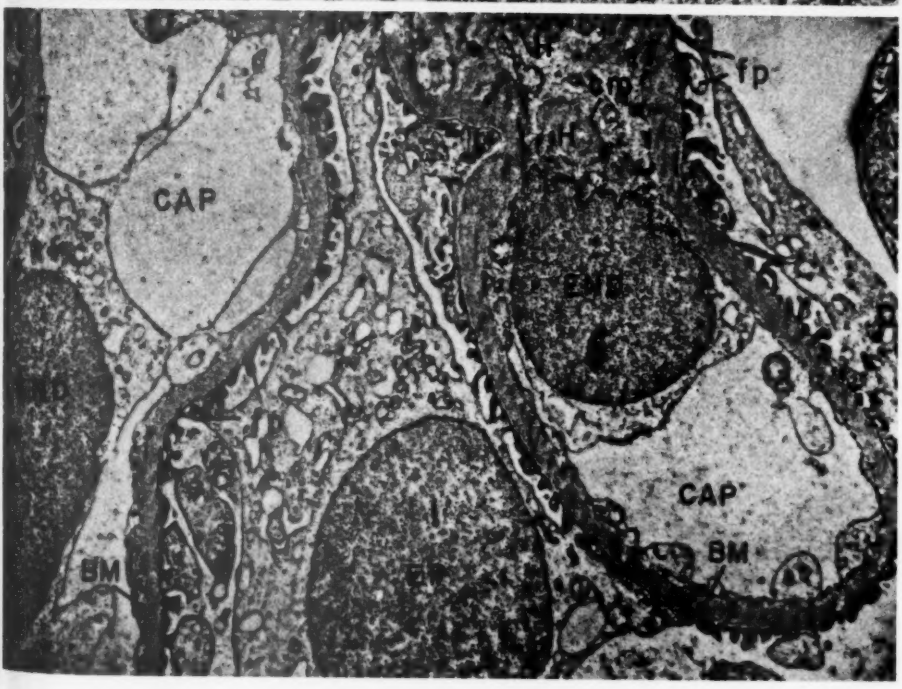

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FIG. 11. Portion of a glomerulus from case 1. This is from the same glomerulus shown in Figure 1. The minimal deposition of pink to purple staining hyalin of the FAH preparation is seen here as a deposit of material similar in appearance to the basement membrane. The hyaline or basement membrane-like material is located between the cell membranes of adjacent endothelial cells and is therefore extracytoplasmic. The axial distribution of the hyaline deposits is shown with the peripheral loops remaining patent. Thickening of the basement membrane to 2 to 3 times normal can also be seen here. This alteration was not evident by light microscopy. The epithelial foot processes the essentially normal. The cytoplasm of both endothelial and epithelial cells shows swelling and disruption in some areas. × 5700.

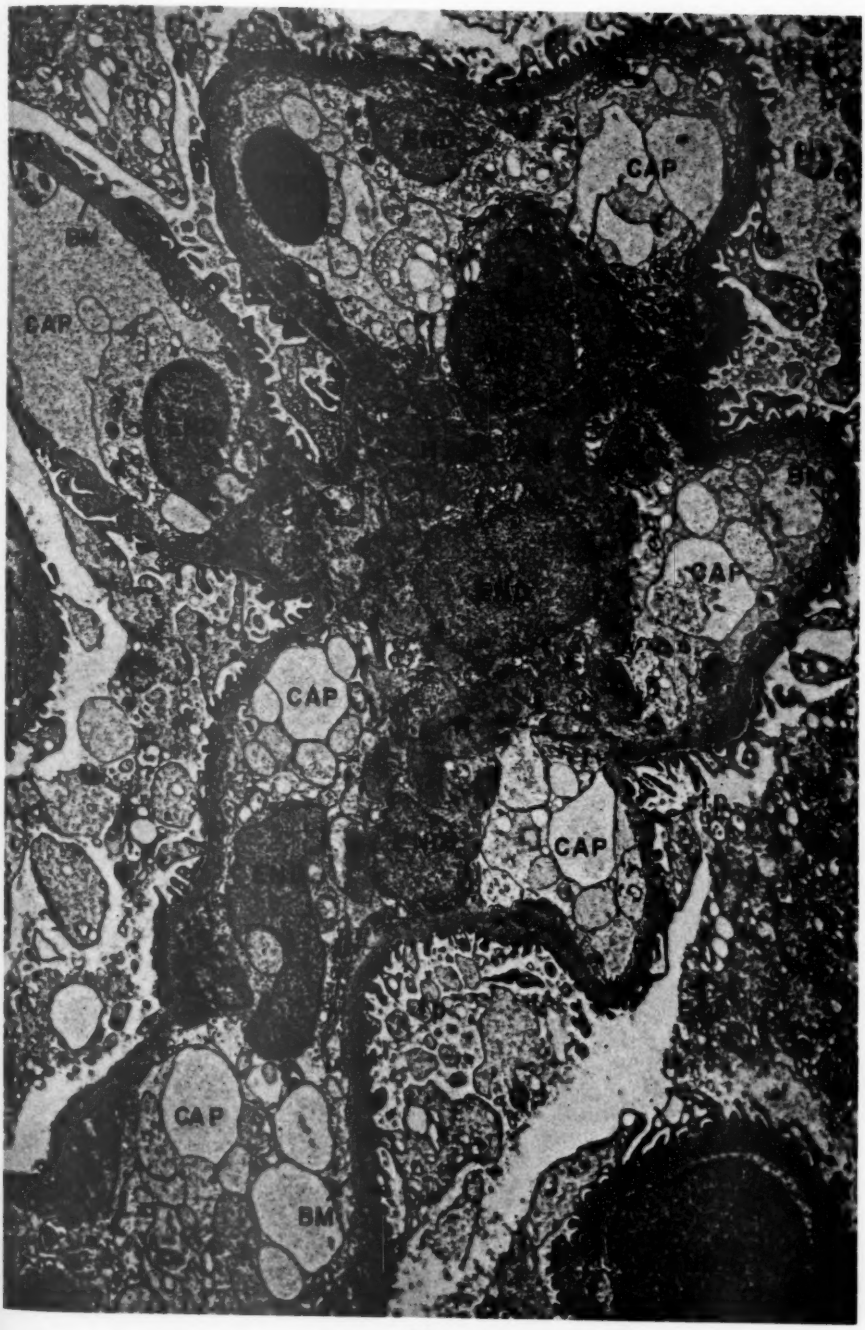

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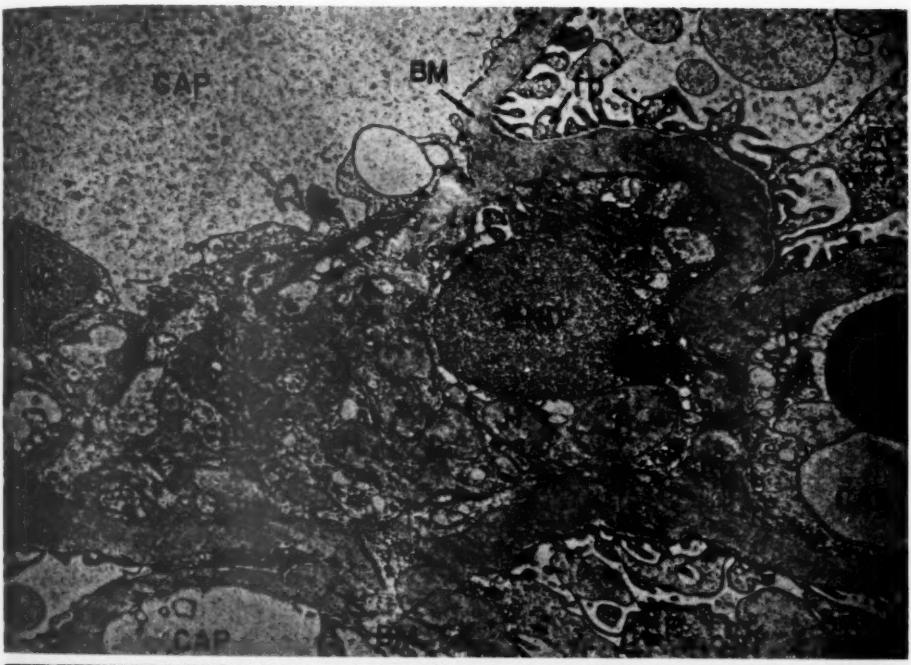
FIG. 12. Another glomerulus from case 1, showing larger amounts of hyaline or basement membrane-like material. An endothelial cell occupies the center of the field. Surrounding the endothelial cytoplasm are a number of relatively small accumulations of basement membrane-like material. This hyalin may be distinguished from the lighter areas of endothelial cytoplasm which contain particulate and vesicular structures. The endothelial cell membranes are seen interposed between endothelial cytoplasm and hyalin except in areas where the cell membrane is presumably sectioned tangentially. The basement membrane shows pronounced thickening to 5 times normal thickness in many areas. The epithelial foot processes appear normal. × 6400.

FIG. 13. Glomerulus from case 4, showing more advanced hyaline deposition. The central portion has been obliterated by hyaline deposits, and only the peripheral loops remain open (see Fig. 3). The epithelial cells show some alteration from the normal, i.e., the foot process layer of cytoplasm is less abundant than normal. In addition, the foot processes have become broad in some areas, resulting in irregularly-spaced interruptions. The cytoplasm of the endothelial cells lining the open loops is thickened and swollen. × 3700.



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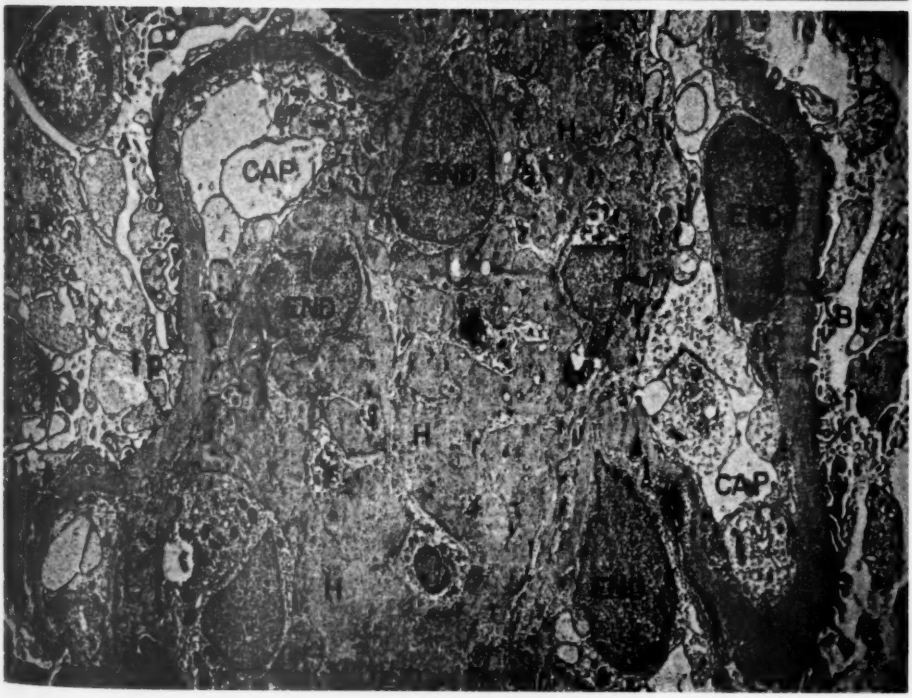

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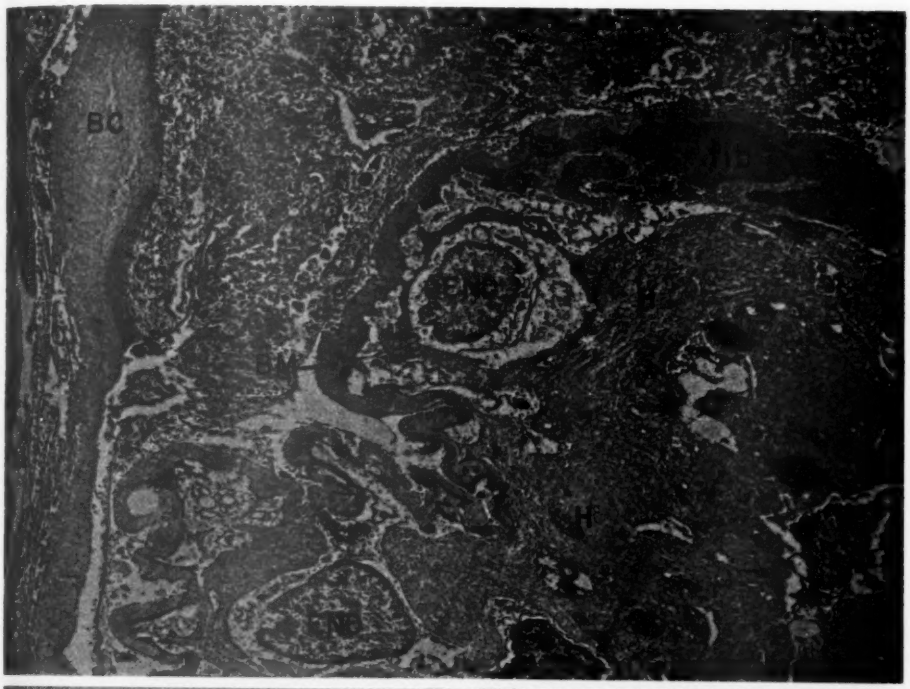
FIG. 14. Glomerulus from case 7. This glomerulus is severely altered by the deposition of hyalin; several endothelial cells with very pale cytoplasm are surrounded by the hyalin. A portion of the glomerular basement membrane can be identified, crossing the field. A segment of the greatly thickened basement membrane of Bowman's capsule is present on the far left. Dense "fibrinoid" of an exudative lesion is present beneath the glomerular basement membrane in the upper right. The "fibrinoid" is very dense compared with hyalin. Exudative lesions are illustrated to better advantage in Figures 15 and 17. At this late stage the hyalin appears more heterogeneous than that illustrated in Figures 9 to 13, for it shows fibrillary structure instead of the relatively homogeneous texture of basement membrane. × 3700.

FIG. 15. Portion of a glomerular loop from case 7, showing minimal fibrinoid of an early exudative lesion. Note that fibrinoid is laid down within the capillary wall between the endothelial cell membrane and the basement membrane proper (i.e., subendothelial). The capillary lumen is patent. The cytoplasm of epithelial cells appears somewhat condensed but shows normal frequency of interruptions into foot processes. The basement membrane is greatly thickened. × 7600.



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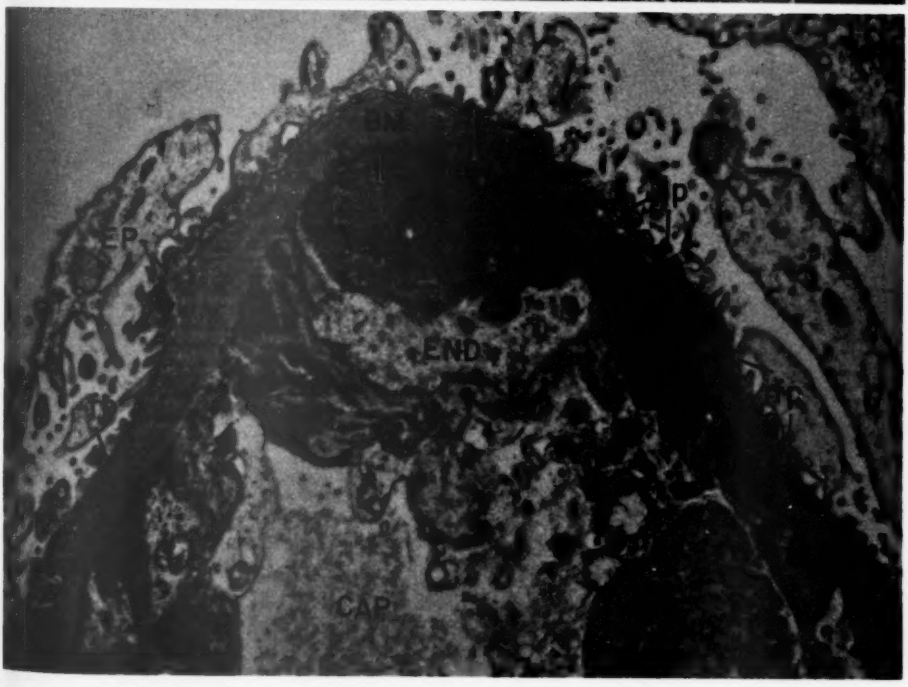

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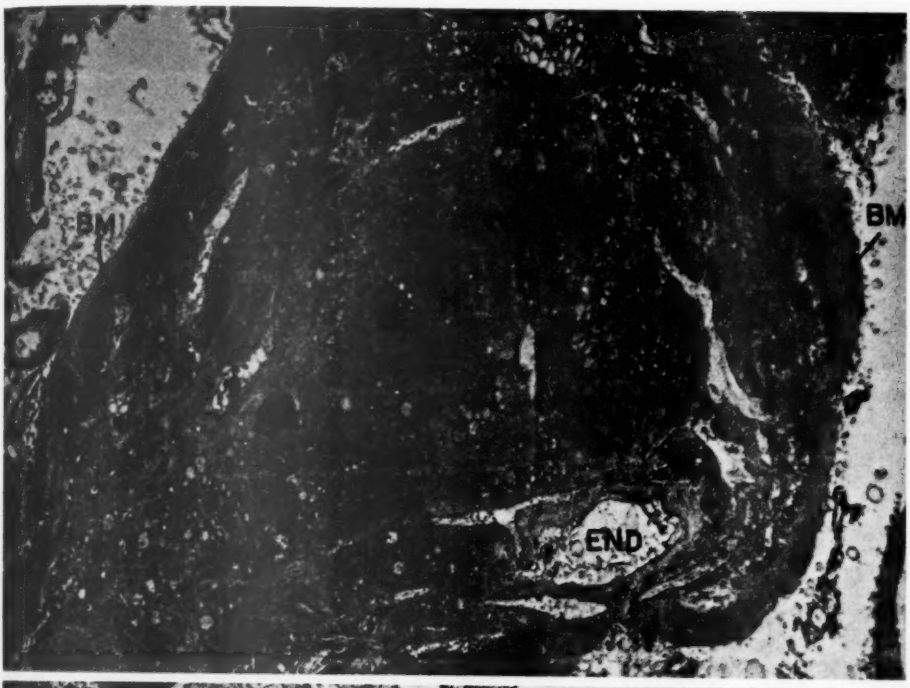
FIG. 16. Portion of another glomerulus from case 7, showing a typical nodular lesion. The only feature which distinguishes the nodular lesion from the diffuse is the size of the hyaline mass. In the diffuse process, hyaline deposition proceeds in a uniform manner at the cell borders of endothelial cells resulting in the isolation of islands of cytoplasm by the expanding mass, as illustrated in Figures 9 to 13. A nodule occurs, as shown here, when the hyaline mass is of a relatively large size; the endothelium is crowded to the periphery, and only occasional cells are trapped within the core. In this late stage of the process, the hyalin shows a more heterogeneous composition than that seen in earlier stages. Isolated areas of fibrinoid deposition or exudative material are also seen beneath the residual basement membrane at the periphery of the nodule. The epithelium is distorted: no foot processes are seen either here or in the following figure. In some areas the epithelial cytoplasm is absent from the outer surface of the basement membrane. × 3600.

FIG. 17. Portion of a glomerulus from case 7, showing several exudative lesions of "fibrin caps" which are composed of relatively large deposits of dense "fibrinoid." The fibrinoid deposits in this glomerulus are subendothelial in position and are found between the thickened basement membrane and the endothelial cell membranes. The accumulation of hyalin centrally and "fibrinoid" more peripherally in these loops has resulted in complete obliteration of the lumens. The appearance of the fibrinoid differs from that of the surrounding hyalin, which is characterized by its lighter density and relatively heterogeneous texture showing fibrillary areas in this severely damaged glomerulus. Foot processes are not seen, and the epithelial cytoplasmic layer is absent from portions of each loop. × 3600.



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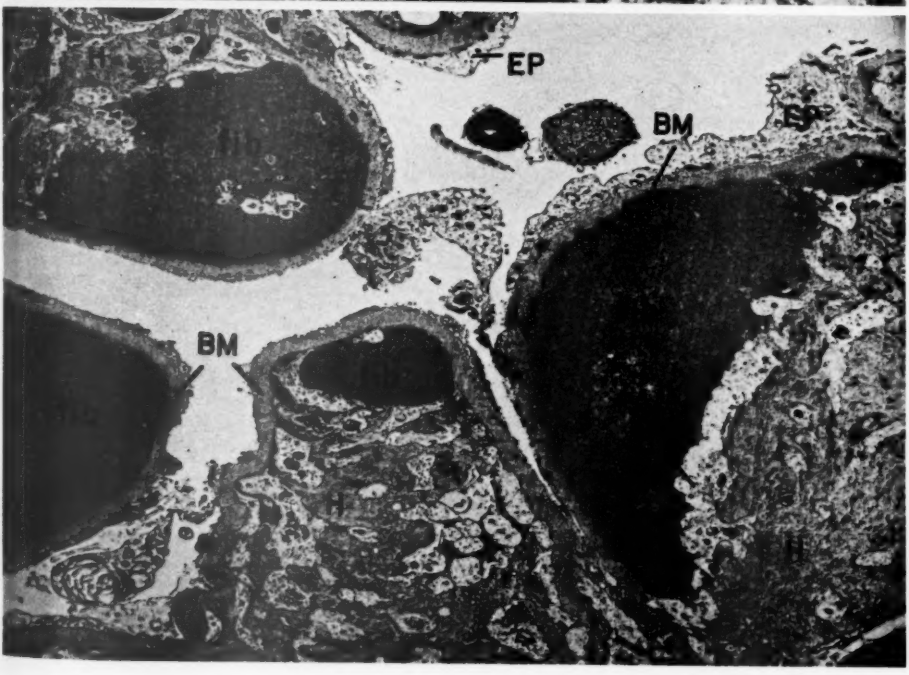

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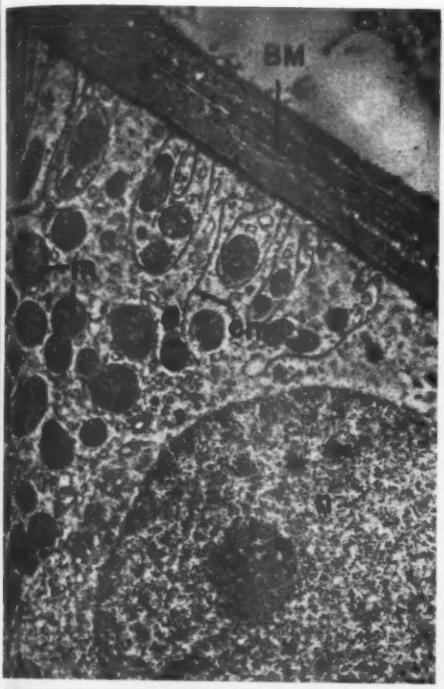
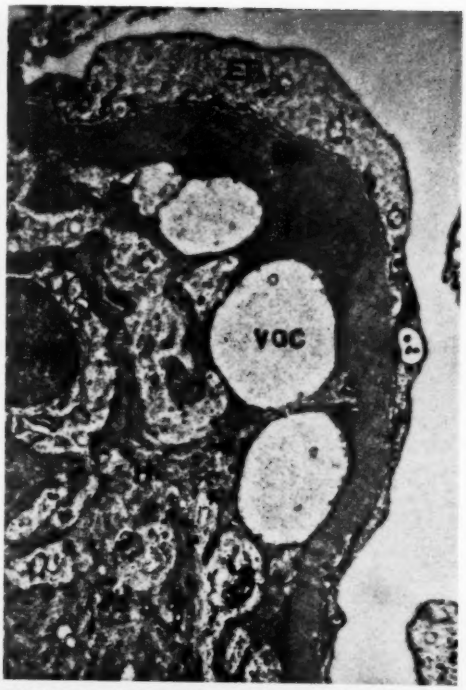
FIG. 18. Portion of the nucleus and cytoplasm of a distal convoluted tubule cell from a patient with diabetes (case 6). A pronounced thickening of the tubular basement membrane is shown. The basement membranes of the tubules are clearly laminated, for they are built up of many parallel layers, in contrast to the relatively homogeneous-appearing glomerular basement membrane. × 6000.

FIG. 19. Portion of a glomerular loop from case 7, showing an exudative lesion which contains several large vacuoles. A loss of the epithelial foot processes with a spreading of the epithelial cytoplasm along the basement membrane is also evident. × 6200.

FIG. 20. Portion of an afferent arteriole of a glomerulus from case 6, showing severe hyaline arteriolosclerosis. The lumen of the arteriole is to the right and the large hyaline deposit to the left. A portion of the cytoplasm of several endothelial cells as well as the internal elastic membrane and several smooth muscle cells are shown. It can be seen that the arteriolar hyalin in this vessel is located external to the internal elastic membrane and partially replaces the smooth muscle layer. To the left of the knife mark, a smooth muscle cell is seen embedded in the hyalin; the latter shows a dense, finely-particulate composition. × 3600.





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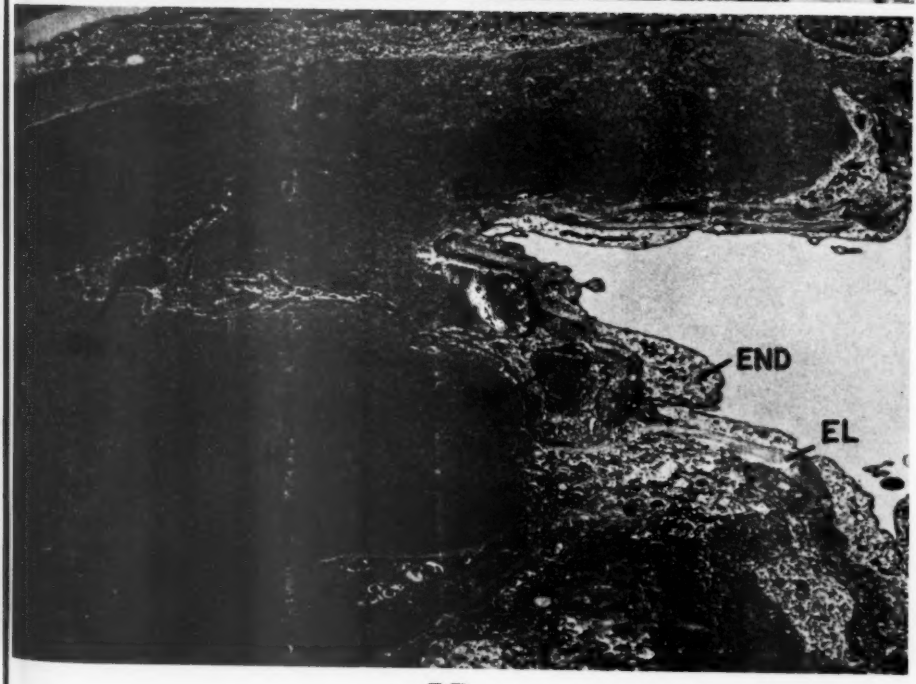

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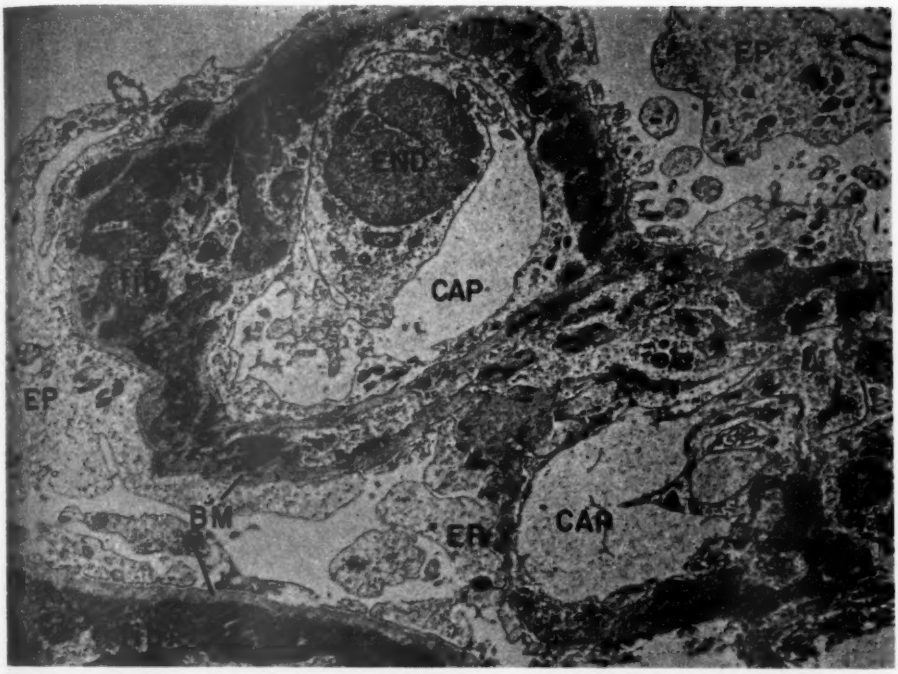
FIG. 21. Portion of the same glomerulus as that illustrated in Figure 7, from a patient with disseminated lupus erythematosus. The chief abnormalities noted in this glomerulus by light microscopy were hypercellularity and the accumulation of fibrinoid in virtually every loop. In this electron micrograph the fibrinoid may readily be distinguished from the remaining glomerular components because of its great density. Like the fibrinoid deposits of the exudative lesions of diabetes, it is predominantly subendothelial in position. However, in several places (e.g., to the left, above) the fibrinoid has apparently permeated the basement membrane and is seen between the latter and the epithelium. The pattern of fibrinoid deposition is quite different from that seen in diabetes. It is also apparent here that the basement membrane is irregularly thickened, and the endothelial cytoplasm appears swollen. Fibrinoid deposition and endothelial swelling have encroached upon the lumen. The organization of the epithelial foot processes is distorted, for there are areas where the epithelial cytoplasm is spread along the basement membrane with only infrequent interruptions. × 5800.

FIG. 22. Portion of a "wire-loop" from a patient with disseminated lupus erythematosus. By electron microscopy, a wire loop lesion consists of a thickened basement membrane proper and a broad subendothelial layer of dense, finely particulate "fibrinoid." The endothelial swelling and distortion of the foot processes seen in Figure 21 are also shown here. × 13,600.



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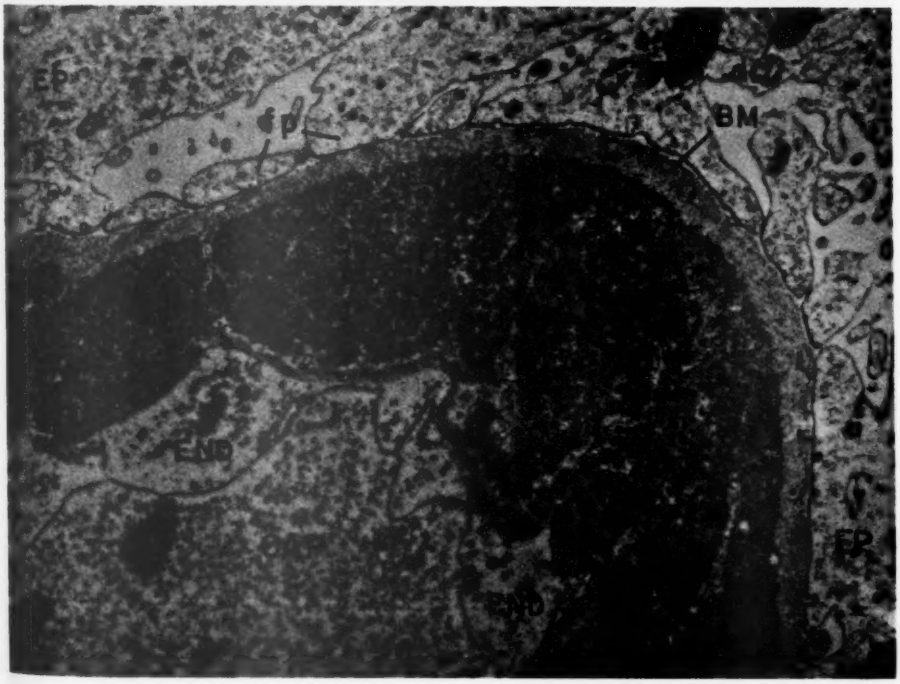

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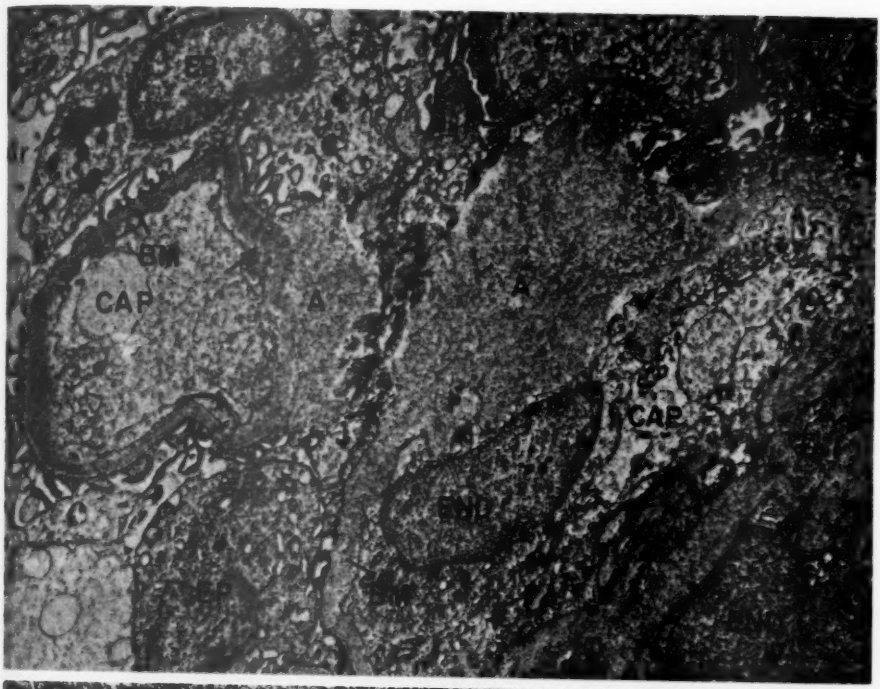
FIG. 23. Portion of a glomerulus from a patient with amyloidosis, showing the alterations in glomerular fine structure which accompany this disease. The basic change is manifested by irregular thickening of the basement membrane proper. Areas of normal basement membrane are present in places, but at several points (arrows) the membrane bulges conspicuously to 20 or more times normal thickness. The thickened areas, presumably representing the amyloid seen by light microscopy after appropriate staining, show a different composition from that of normal basement membrane: they are lighter in density and have a "foamy" texture at low magnification. At higher magnification tiny vesicles and tubules make up the background of the amyloid. × 11,000.

FIG. 24. Glomerular loop from a patient with pre-eclampsia. There is a pronounced swelling of the endothelial cytoplasm resulting in almost complete occlusion of the lumen. Arrows outline the greatly restricted residual lumen. Subendothelial fibrinoid deposits are seen surrounding the loop, and some fibrinoid is also present between the cell membranes of adjacent endothelial cells. There is also an increase in the amount of epithelial cytoplasm. The basement membrane appears to be of normal thickness. × 4000.



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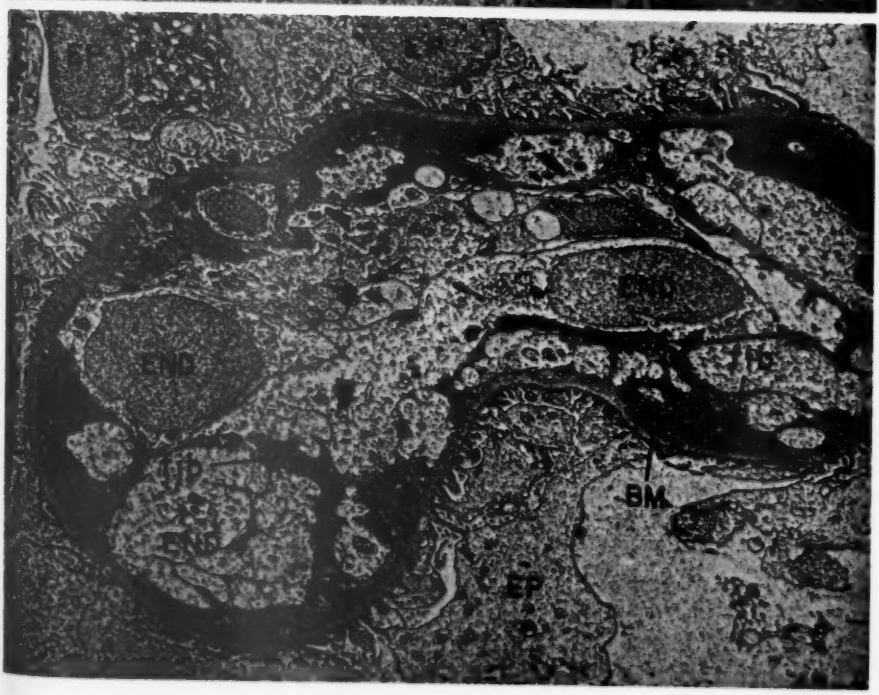

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HISTOLOGIC ASPECTS OF SUBACUTE GLOMERULONEPHRITIS; WITH SPECIAL REFERENCE TO PROLIFERATIVE ALTERATIONS IN THE EPITHELIUM OF RENAL TUBULES *

URIEL GARCÍA-CÁCERES, M.D.
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Diffuse glomerulonephritis with epithelial crescents in Bowman's capsule is classically designated as "subacute." This term is also applied, with some resulting confusion, to several forms of progressive and rapidly fatal glomerulonephritis in which crescents are not necessarily encountered.[1] It has been thought that the crescents identify one stage in the development of glomerulonephritis as it passes from the acute to the chronic phase.[2,3]

The glomerular crescents have been and still are considered a result of proliferation of one component of the nephron unit, the expanded proximal end of the renal tubule, Bowman's capsule.[1-3] One important feature in the renal lesion in this condition has been generally overlooked. This is characterized by a distinctive and constant participation in the proliferative process by the epithelium in the proximal convoluted tubules, resulting in the formation of "increscences," identical in structure to the crescents. There is also an accompanying hyperplasia of characteristic form in the tubular epithelium at a distance from the glomerulus. The observation of these phenomena has stimulated a review of the histologic features in a number of examples of subacute glomerulonephritis.

MATERIAL AND METHODS

Five cases of subacute glomerulonephritis with crescent formation were found in the slide files of necropsy examinations carried out at the Instituto de Anatomía Patológica, San Marcos University Faculty of Medicine, between 1948 and 1957. Each of these exhibited the lesions which serve as the basis for this report. In addition, microscopic sections from 42 cases of subacute glomerulonephritis listed in the files of the Section of Pathological Anatomy at the Mayo Clinic† between 1940 and 1957 were examined. Twenty-two (52 per cent) were found to exhibit capsular crescents; this lesion was absent in the

* Received for publication, December 29, 1958.

† This portion of the investigation was carried out during a 4-week period of study at the Section of Pathological Anatomy of the Mayo Clinic, Rochester, Minn., under a Traveling Fellowship Grant provided by the Kellogg Foundation, Battle Creek, Mich.

remainder, in which the diagnosis of subacute glomerulonephritis was based upon other criteria.[1]

The microscopic examination of specimens from the 27 cases with crescent formation was carried out on sections as they were available in the files. In each instance there was at least one section from each kidney; the majority had more. All sections were stained with hematoxylin and eosin; about half were stained by the Mallory-Heidenhain and periodic acid-Schiff (PAS) methods.

The following histologic features were sought and quantitated: (a) Bowman's capsule crescents: (b) proximal convoluted tubular "increscences"; (c) tubular epithelial hyperplasia; and (d) mitotic activity in the tubular epithelium at a distance from the glomeruli. The results are shown in Table I.

Results

The recognition of tubular "increscences" was based upon the ingrowth of epithelial elements into a glomerular space from its related proximal tubule (Figs. 1 to 4, and 8). A co-existing feature of characteristic intratubular epithelial projections was evident in cross sections of juxtaglomerular tubules (Figs. 5 to 7). In the latter lesion the outer limits of the tubular basement membrane were clearly evident.

Increscences were composed of cells arising from tubular rather than capsular epithelium. They were made up of tufts of cells arranged without discernible order, which often completely occluded the lumen of the related tubule. If the plane of the section was appropriate, the increscence seemed to extend into the tubule from the capsular crescent in such a fashion as to afford a tadpole-like appearance (Figs. 1 to 4, and 8). The proliferating cells were spindle shaped and, within the capsular space, were arranged parallel to the outline of the capsule. In the tubule, on the other hand, they grew without organized polarity. In occluded tubules, the growth was characterized by tufts arising from 2 or 3 separate loci, between which the original tubular epithelium was still visible (Fig. 5). More often, however, the proliferation was uniform about the entire circumference of the tubule (Fig. 7).

Mallory-Heidenhain and PAS stains demonstrated a delicate framework of connective tissue fibrils between the cells of the increscences. The mesh was identical in appearance to that seen in glomerular crescents, and appeared to arise from the basement membrane (Fig. 8). At times, when scarring had taken place, there were fibroblasts intermixed with the epithelium (Figs. 2, 3 and 8). In this instance the related glomeruli usually showed adhesions between the capillary loops

TABLE I

Histologic Features Observed in 27 Cases of Subacute Glomerulonephritis

Case no.*	Glomerular lesion	Bowman's capsule crescent†	Convoluted tubular increscence†	Tubular epithelial hyperplasia‡	Mitotic activity§
1	Chronic sclerosing	+++	+++	+++	++
2	Proliferative	++	+	+++	++
3	Proliferative and exudative	+++	++	+++	+
4	Proliferative	+++	++	+++	++
5	Chronic sclerosing	+	+	++	o
6	Proliferative and exudative	+++	+++	+++	++
7	Proliferative	+++	++	+++	
8	Proliferative	++	+	+	o
9	Acute necrotizing	+++	+++	+++	++
10	Chronic sclerosing	+	+	+++	+
11	Chronic sclerosing	+	+	+	o
12	Chronic sclerosing	++	+++	+	+
13	Acute proliferative**	+++	+++	+++	+++
14	Proliferative	++	+	+++	
15	Proliferative	++	++	+++	+
16	Proliferative	+++	+++	+++	++
17	Chronic sclerosing	+	+	++	o
18	Proliferative and exudative	+	+	+++	++
19	Proliferative	+	+	+	+
20	Proliferative	++	++	+++	++
21	Proliferative and exudative	+	+	+	+
22	Chronic sclerosing	+	+	+++	+
23	Chronic sclerosing	++	+	++	o
24	Proliferative	+	+	+	o
25	Proliferative	++	++	+++	+
26	Proliferative	+++	+++	+++	++
27	Chronic sclerosing	+	+	+	+

* The first 5 cases are from the files of Instituto de Anatomía Patológica, University of San Marcos Faculty of Medicine. The remainder are from the Mayo Clinic, Rochester, Minn.

† Estimation of characteristics was based on the following:
+ = less than 25 per cent of the glomeruli and tubules examined contained the lesion.
++ = lesion manifest in approximately 50 per cent.
+++ = lesion present in 75 per cent or more.

‡ The tubular epithelial hyperplasia was graded upon the following criteria:
+ = dilatation of tubules, flattening of epithelium and occasional cells with double nuclei.
++ = dilatation of tubules, flattening of epithelium and many cells with double or triple nuclei.
+++ = dilatation of tubules, flattening of epithelium, cells with double or triple nuclei in most of the tubules and also buds of epithelial hyperplasia.

§ Mitotic activity was graded as follows:
+ = one mitotic figure per 10 high power fields.
++ = one mitotic figure per 5 high power fields.
+++ = one mitotic figure in fewer than 5 high power fields.

** The designation "acute proliferative" indicates the most striking example of glomerular proliferation.

and capsule. Evidence of acute glomerular inflammation characterized by exudate and necrosis was invariably accompanied by similar alterations in both capsular crescents and tubular increscences.

Artifacts, such as desquamated cells or tangentially sectioned glomeruli, were distinguished from tubular increscences with ease. Moreover, there appeared to be no need for serial sectioning, since in a single preparation, connections between tubules and glomeruli were seen in various planes (Figs. 1 to 4, and 6). This, of course, does not exclude the necessity for such a study, if only to demonstrate the distance that the increscent proliferation extends into the tubule. In the present study, tubular epithelial proliferation was observed to extend for distances up to 1 mm. into the proximal tubules.

All examples of subacute glomerulonephritis with crescent formation exhibited unequivocal evidence of tubular increscence. However, correlation between the severity of crescent and increscence production was not possible since serial sections were not made.

Hyperplasia of the tubular epithelium was a constant concomitant. Tubular epithelial cells often contained double nuclei with prominent nucleoli (Fig. 10). These were quite different from the giant cells with pyknotic nuclei described by Harman and Hogan[4] in association with a variety of conditions. Mitotic figures were readily observed (Fig. 9). In some instances hyperplasia was so great that there was an apparent overlapping of nuclei, and in many instances the epithelium was multilayered (Figs. 4 to 6). The proximal and distal segments were difficult to differentiate after the lining cells had increased in number and the lumens had dilated.

Glomerular lesions were classified in accordance with the current nomenclature (Table I). It was found that 21 per cent were examples of acute necrotizing glomerulitis with exudate; 43 per cent exhibited proliferative glomerulitis; and 36 per cent showed chronic sclerosing alteration. Although in the majority an advanced degree of obliteration of glomerular architecture had not occurred, there was, nonetheless, obvious scarring.

Discussion

This investigation would appear to indicate that proliferation of the parietal layer of Bowman's capsule with crescent formation is invariably accompanied by similar proliferation of the tubular epithelium and the formation of increscences. A search of the pertinent literature has failed to elicit mention of lesions resembling tubular increscence. Passing reference to proliferative activity of tubular epithelium in the crescent type of glomerulonephritis,[5] apparently unlike increscence

formation, indicates that it has been regarded as a phenomenon secondary to tubular necrosis. Older reports reflect a clear awareness of the fact that in some instances of nephritis there was a prominent hyperplasia of the tubular epithelium.[2,6-8] Oertel,[7] for example, spoke of *"nephritis prolifera"* as a special type of Bright's disease in which proliferation of the tubular epithelium was a prominent feature. However, there was no attempt to link this with the epithelial proliferation of Bowman's capsule or lesions resembling increscences.

In more recent years, tubular alterations accompanying all forms of glomerulonephritis were considered to be secondary to glomerular ischemia and to be of atrophic nature,[1-9] in spite of the studies of Oliver and co-workers[10,11] and others[12,13] demonstrating a prominent elongation of the proximal convoluted tubules in chronic forms of nephritis. Indeed, one might suspect that a good part of the increase in size and weight of the kidneys in subacute glomerulonephritis may well be due to tubular hyperplasia.

Eisen in 1946[14] described an instance of "adenomatoid transformation of the glomerular capsular epithelium" in a patient with carcinoma of the gallbladder who died in renal failure. The lesion was considered to be a peculiar, diffuse, tumor-like proliferation of the parietal layer of Bowman's capsule with ". . . occasional extension, for a short distance only, into some of the proximal convoluted tubules." From the illustrations in this paper one may suspect that this lesion was an unrecognized example of proliferative glomerulonephritis.

There is probably no other condition in which the tubular epithelium shows such a degree of activity. Regularly there are binucleated cells, overlapping of cells, and mitotic activity of an order rarely encountered in the most severe examples of necrotizing tubular disorders. Moreover, tubular epithelial necrosis is not a feature of this condition. The epithelial regeneration observed in some instances of "lower nephron nephrosis" (tubulorhexis) is characterized by low cuboidal epithelium, multinucleated epithelial elements and dilatation of tubules. These features bear only a remote resemblance to those encountered in glomerulonephritis.

The glomerular capillaries in this form of glomerulonephritis do not exhibit a pathognomonic lesion.[15,16] This fact has been variously interpreted by Volhard and Fahr[2] and others.[1,17] The most common feature is a proliferation of cells which, according to the electron microscopists, are probably of histiocytic origin.[18,19]

The present investigation indicates that the crescents, increscences and tubular epithelial hyperplasia constitute complications occurring

at any time in the course of glomerulonephritis, regardless of the type of glomerular lesion. In support of this concept are two cases in which the time of onset of the glomerulonephritis was reasonably clearly established. In case 14 (Table I), a nephrectomy was carried out because of gross hematuria incorrectly attributed to a surgical lesion; the patient died two months later. The resected kidney showed only mild glomerulonephritis with slight proliferative activity of the tubular epithelium and occasional crescents; two months later, the other kidney showed diffuse crescent and increscence formation. In case 1 (Table I), the symptoms were clearly of two years' duration. Following infection in a compound fracture, albuminuria was noted for the first time. The clinical symptoms thereafter were those indicating progression to the full blown syndrome of Bright's disease. The glomerular lesion observed at necropsy in this patient was characterized by crescent and increscence formation and was thought to represent that termed by Allen[1] as "chronic sclerosing glomerulonephritis" and by Volhard and Fahr as *"Kombinationsform."*[2]

There is no satisfactory explanation for the formation of crescents, increscences, or the existence of tubular epithelial proliferation. Assuming that all 3 alterations have a common origin, the explanations given for either the crescentic proliferation or the tubular epithelial hyperplasia may be invoked to explain all 3.

The consensus of opinion on the pathogenesis of the epithelial crescents has been restated by Rich[20] who reported that the capsular epithelium might proliferate over and into a coagulum of blood or exudate in the glomerular space. This also may be the case in the formation of increscences; however, the association of clots of blood or protein with either form of proliferation (crescents and increscences) is by no means constant.

As far as tubular epithelial proliferation is concerned, the hypothesis of Oertel,[7] explaining the pathogenesis of *"nephritis prolifera"* by epithelial proliferation as a special form of inflammatory reaction localized to these cells, can no longer be substantiated. This was based upon Virchow's[21] concept of catarrhal inflammation. An alternative suggestion is that the activity of the tubular epithelium may be regenerative in nature.[1] However, cellular necrosis is not a prominent feature; on the other hand, there are many instances of acute and chronic glomerulonephritis with marked interference with the circulation in the glomerular tufts, but no epithelial proliferation.

It is possible that electrolyte imbalance, so commonly associated

with glomerulonephritis, may explain the peculiar proliferative tubular lesion. The information available is, as yet, meager and contradictory.[22-28] However, Oliver and co-workers[29] have recently shown that marked epithelial proliferation can be induced in the proximal convoluted tubule of the rat by potassium depletion. On the other hand, no publications have been found indicating experimental study or clinicopathologic observations correlating proliferative phenomena in the renal tubular epithelium of glomerulonephritis with electrolyte alterations.

Summary

The histologic aspects of glomerulonephritis associated with the formation of capsular epithelial crescents (subacute glomerulonephritis) have been reviewed. Evidence is presented to show that the crescentic proliferation is constantly associated with a heretofore unrecognized proliferation of the proximal convoluted tubular epithelium. This is characterized by "increscence" formation and epithelial hyperplasia in the proximal convoluted tubules.

There is wide variation in the structure of the associated glomerular lesion. This would indicate that the development of crescents, "increscences" and tubular epithelial hyperplasia may occur at any time in the course of glomerulonephritis.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Case 3. Glomerular crescent and tubular increscence. The plane of the section is such that it shows only a few glomerular capillaries. Note the direct relationship of the proliferating epithelial cells of Bowman's capsule and the proximal convoluted tubule. Hematoxylin and eoson stain. × 300.

FIG. 2. Case 1. Glomerular crescent and tubular increscence. Note the scarring in the periglomerular space and the glomerular capillaries. This case has a clearly established duration of 2 years. Hematoxylin and eosin stain. × 300.

FIG. 3. Case 5. The glomerular tuft shows atrophy. There are numerous adhesions at one side of the capsule. The proximal convoluted tubule is emerging directly and shows the increscence up to the point of bending. Figures 1 to 4 and 8 show a "tadpole" appearance of the lesion in Bowman's capsule and the tubule. Hematoxylin and eosin stain. × 300.

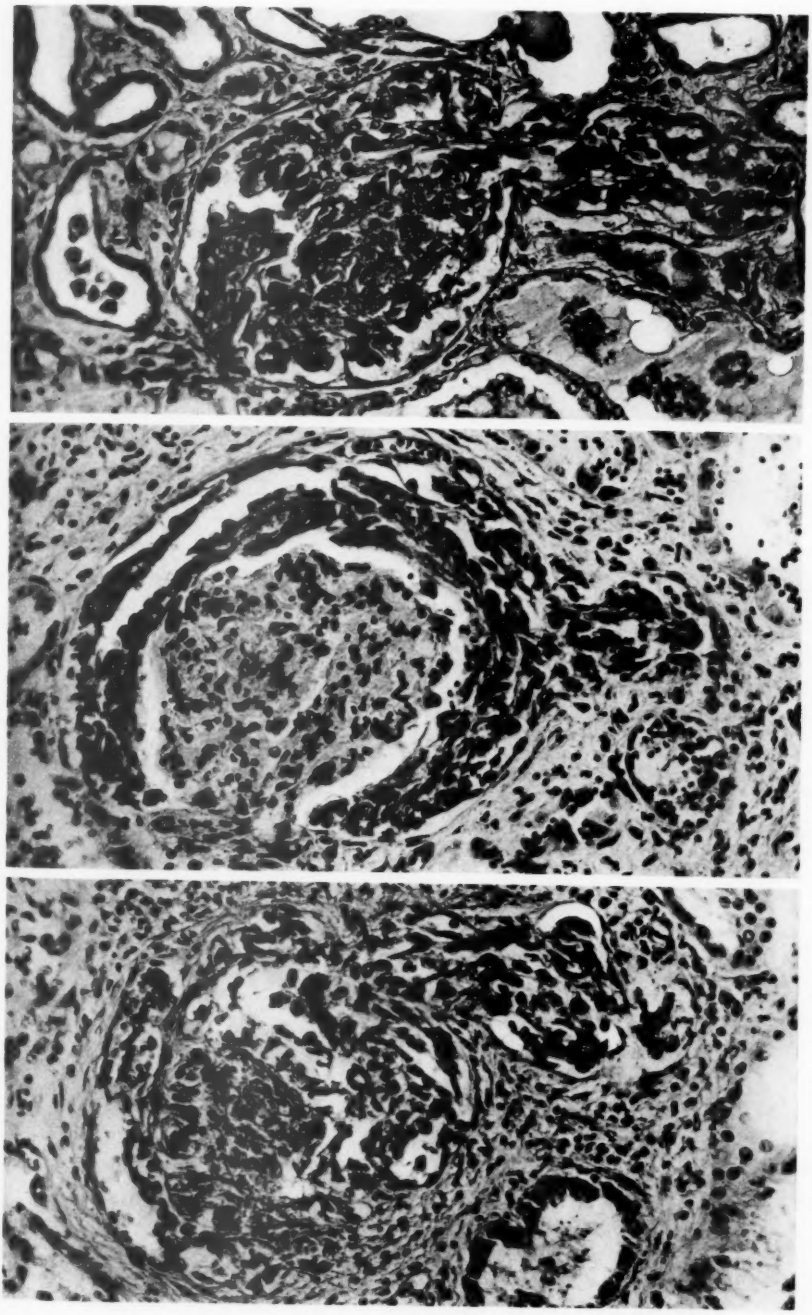

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FIG. 4. Case 26. The tubular increscence, seen longitudinally and transversely, coming off the glomerular space. The epithelial proliferation (increscence) is evident up to the point of bending of the proximal convoluted tubule and still is visible in the next cross section. There is prominent hyperplasia of the tubular epithelium with overlapping of nuclei and double and triple nucleated cells. Hematoxylin and eosin stain. × 225.

FIG. 5. Case 23. Cross section of a tubular increscence in a juxtaglomerular area. There are 2 different loci of growth between which the original epithelium is still visible. Hematoxylin and eosin stain. × 700.

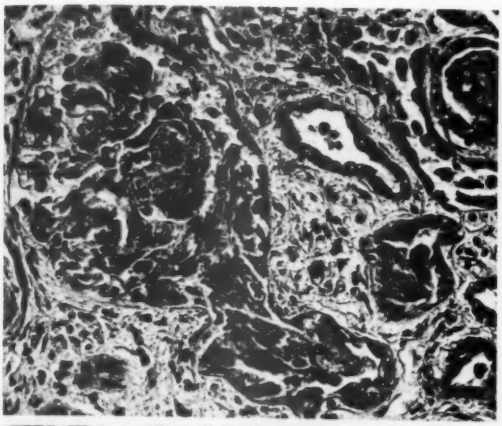
FIG. 6. Case 7. Longitudinal section of a juxtaglomerular tubule with increscence formation. The glomerulus is seen at one side. All sorts of transitions between the proximal portion of the tubules and Bowman's capsule can be seen. Hematoxylin and eosin stain. × 275.

FIG. 7. Case 20. Transverse section of a glomerular increscence with uniform proliferation about the entire circumference of the tubule. Compare with Figure 5. Hematoxylin and eosin stain. × 575.

FIG. 8. Case 5. The connective tissue fibrils are identical in both the glomerular crescent and the tubular increscence. They seem to grow from the basement membrane. Periodic acid-Schiff stain. × 300.

FIG. 9. Case 18. Tubular epithelial hyperplasia. Two cells in mitosis in a tubule with an increase in number of cells and widening of the lumen. At this stage it is most difficult to differentiate proximal and distal tubules unless they are coming off directly from a capsular space. Hematoxylin and eosin stain. × 535.

FIG. 10. Case 10. A cell with double nuclei in the lining of a tubule. The nucleoli are prominent. They are quite different from the multinucleated cells with pyknotic nuclei described by other authors. Hematoxylin and eosin stain. × 900.



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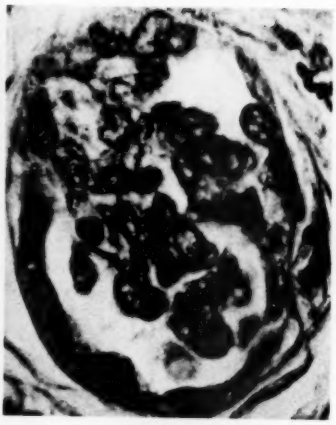

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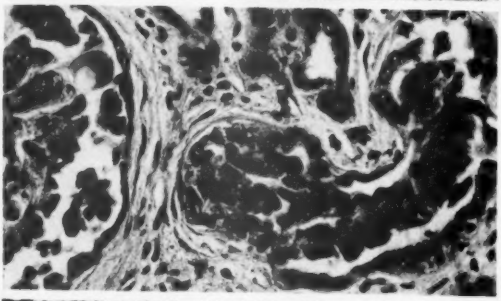

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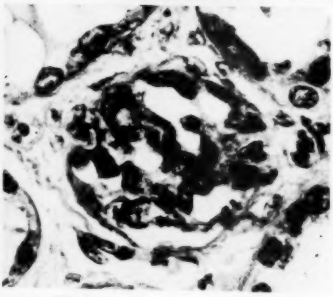

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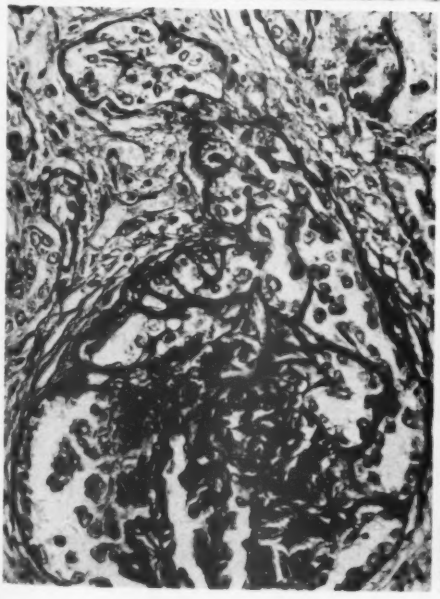

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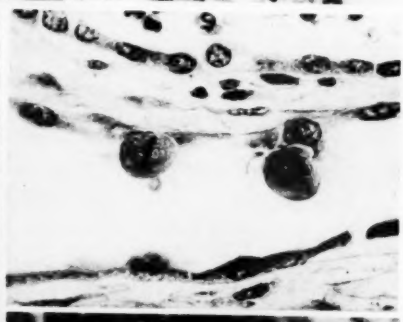

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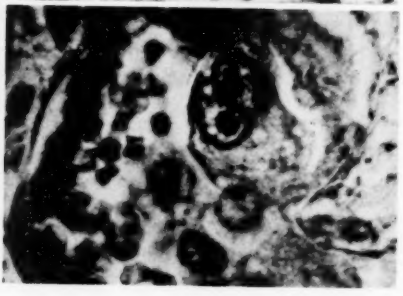

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WIDELY DISTRIBUTED NECROTIZING ARTERITIS INDUCED IN RABBITS BY EXPERIMENTAL RENAL ALTERATIONS.

II. Relationship of the Arterial Lesions to Perirenal Inflammation *

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In a previous communication[1] it was reported that widely distributed necrotizing arteritis and gradually rising hypertension were consistently induced in rabbits by wrapping the left kidney in silk saturated with turpentine and performing right nephrectomy 7 days later. Further experimentation has shown that an even more rapidly rising hypertension may be induced in the absence of arteritis by increasing the interval from 7 days to several months between the wrapping of one kidney with turpentine-saturated silk and the removal of the contralateral kidney. Furthermore, in a supplementary experiment in rabbits, a prompt and sustained elevation of blood pressure which followed bilateral carotico-aortic denervation was likewise not attended by the occurrence of arterial lesions.

The purpose of this paper is to report on the dissociation of rapidly rising blood pressure in rabbits and the development of necrotizing arterial lesions under certain experimental conditions. It is further shown that the development of necrotizing arterial lesions is related essentially to the existence of acute unilateral exudative perinephritis and the absence of a contralateral kidney.

Material and Methods

Fifty-one rabbits, both males and females, ranging from 4 months to one year in age, comprised the experimental group. Forty-four of the rabbits were from a stock bred at the Rockefeller Institute for Medical Research, New York. The remainder were from the Zartman Commercial Breeding Farm of Douglassville, Pennsylvania. The animals were fed Rockland rabbit pellets and received water *ad libitum*. Pieces of thin white Japanese silk and steam distilled wood turpentine (Alpha brand, George Isaacs and Company, Inc., New York and South Kearny, New Jersey) were used. For anesthesia and sacrifice of the animals, veterinary pentobarbital sodium (Abbott) was used.

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Morphologic Methods

The cardiac index has been previously defined[1] by the following formula: $\frac{\text{heart weight}}{\text{carcass weight}}$. The carcass weight is that measured after removal of the brain, pituitary, lungs, heart, thymus, spleen, pancreas, gonads, aorta, alimentary tract, and small blocks of other tissues.

"Standard" microscopic examination of sections stained with hematoxylin and eosin was carried out on the following: a coronal section of the cerebral hemispheres; a section through the cerebellum and the pons; one through the right ventricle and the interventricular septum of the heart; two from the left ventricle through each papillary muscle; two from the lung, pancreas, adrenal, colon, gonad, and kidney; and one section each of spleen, esophagus, stomach, duodenum, jejunum, ileum, cecum, diaphragm, quadriceps femoris, ear, and liver with gall bladder.

The vascular lesions observed were classified as "early" and "late," as defined in detail elsewhere,[1] and counted. The average total count of both early and late lesions was utilized to evaluate the overall severity of vascular damage. The average ratio of early to late lesions was graphed as a function of time (Text-fig. 2).

Bacteriologic Investigation

Bacteriologic cultures on both Todd-Hewitt Neo-peptone broth and blood agar plates were made at necropsy examination from the blood of the inferior vena cava in all animals and from all surgically manipulated perirenal areas.

Blood Urea Nitrogen

Blood was obtained prior to operative procedures and before death. Serums from these specimens were stored at 4° C., and determinations of the serum urea nitrogen were carried out according to the method of Van Slyke and Cullen[2,3] at intervals varying from a few days to 6 months.

Blood Pressure Studies

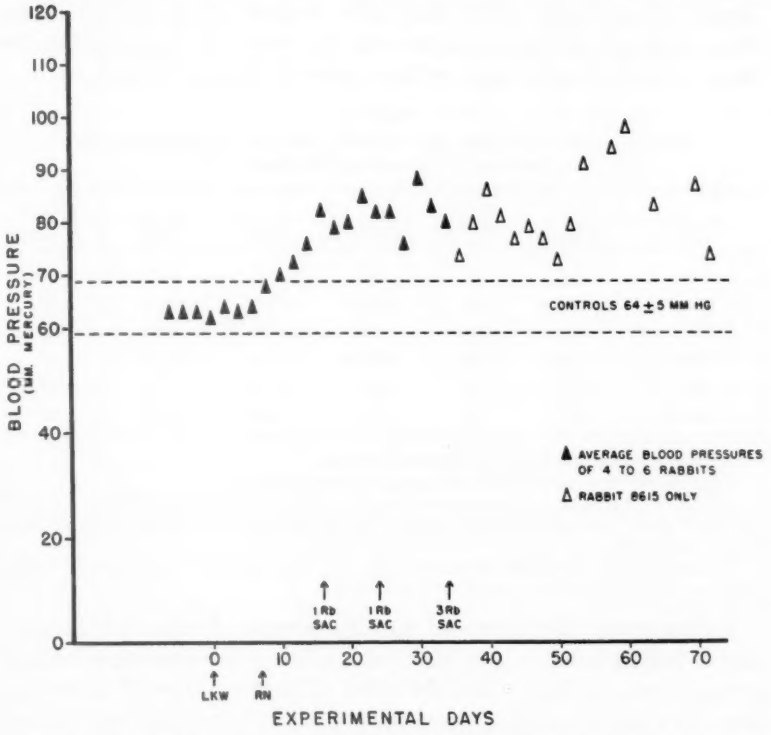
A modification of the Grant-Rothschild capsule was used in the determination of blood pressure. The method has been reported in a previous communication;[1] briefly, the apparatus measures the pressure in mm. of Hg necessary to collapse the central ear artery. On each day that the blood pressure was measured, the values of 4 readings, usually 2 from each ear, were taken; and values obtained on 2 consecutive days were then averaged and graphed.

Experimental Procedures and Results

Acute Perinephritis Followed by Unilateral Nephrectomy

In the experiment to be described below we utilized aseptic surgical techniques in the wrapping and surgical removal of kidneys so that allergic reaction to micro-organisms or direct injury by their toxic products would not play a role in the development of vascular lesions.[4-7]

Utilization of Dry Silk. The left kidney in 6 rabbits was wrapped in thin silk, according to the method described elsewhere,[1] and the right kidney was removed 6 to 8 days later. From the day of production of the silk perinephritis, the rabbits were sacrificed as follows: 1 rabbit at 15 days; 1 at 24 days; 1 at 34 days; 1 at 35 days; 1 at 36 days; and



Text-figure 1. Blood pressure observed after the left kidney was wrapped in dry silk (LKW) followed in 7 days by right nephrectomy (RN). Following right nephrectomy, blood pressures rose gradually in all animals. The "standard" histologic examination showed varying numbers of necrotizing lesions in the small arteries and arterioles of the alimentary tract, gall bladder, liver, testis and heart. Rabbit 8615 was sacrificed 306 days after the induction of the silk perinephritis.

1 at 306 days. Blood pressure readings were taken every day or every other day throughout the experiment.

Results. The blood pressure rose progressively after nephrectomy in all animals (Text-fig. 1). Thorough histologic examination of the alimentary tract disclosed necrotizing lesions in small arteries and arterioles (Figs. 1 and 5). A few of the arteries and arterioles of the heart, liver, gall bladder, and testis in some animals were also injured. The number of lesions found in the "standard" microscopic examination in each animal is given in Table I.

Cultures of the blood and perirenal regions yielded no growth. The cardiac indices in 4 of 6 animals were greater than the normal control range of 2.0 to 3.3 $\times$ 10^{-3}.[1] Terminally only 3 of the animals had blood urea nitrogen values that were elevated above normal range. No correlation was apparent between the degree of hypertension, the level of blood urea nitrogen, or the number of vascular lesions.

TABLE I

*Necrotizing Arterial Lesions after Unilateral Dry Silk-induced Perinephritis Followed by Contralateral Nephrectomy**

Rabbit no.	Day of sacrifice†	No. of arterial lesions	Ratio of early to late lesions‡	Terminal BUN§	Cardiac index§	Maximum blood pressure§
8909	15	12	12/0	28	3.70	81
8907	24	93	67/26	25	3.24	93
8748	34	16	15/1	26	4.01	103
8908	35	53	18/35	50	4.50	96
8815	36	8	3/5	79	4.14	101
8615	306	1	0/1	40	3.18	97

* Blood pressure rose gradually after nephrectomy.

† Dated from time silk perinephritis operation was performed.

‡ Early arterial lesions showed necrosis, nuclear pyknosis or exudative inflammation of the vessel wall. Late arterial lesions showed young, organizing connective tissue or scars in the vessel wall.

§ Control levels: Blood urea nitrogen (BUN), 23 $\pm$ 4 mg. per 100 ml.; cardiac index, 2.0 to 3.3 $\times$ 10^{-3}; blood pressure, 64 $\pm$ 5 mm. of Hg.

Utilization of Silk Saturated with Turpentine. Because of the paucity of lesions found in 4 of the 6 rabbits in the previous experimental group, it was decided to test the effect of greater perirenal inflammation. To achieve a more intense inflammation, turpentine was chosen as an inflammatory agent.

The left kidney in 23 rabbits was wrapped in the manner described previously.[1] Three cc. of sterile turpentine were slowly dropped over the wrapped kidney, and the organ was replaced in its original posi-

TABLE II

*Necrotizing Arterial Lesions after Unilateral Perinephritis Induced by Turpentine-soaked Silk Followed by Contralateral Nephrectomy**

Rabbit no.	Day of death or sacrifice†	No. of arterial lesions	Ratio of early to late lesions‡	Terminal BUN§	Cardiac index§	Maximum blood pressure§	Experimental day of maximum blood pressure
8683 (a)	4	0	0	—	—	63	—
8687 (a)	4	0	0	—	—	67	—
8686 (a)	8	0	0	—	—	68	—
8684 (a)	9	0	0	—	—	64	—
8854 (a)	10	0	0	—	3.06	68	—
8855 (s)	15	22	18/4	37	3.28	87	14
8856 (s)	15	40	39/1	49**	3.28	83	12
8910 (s)	15	36	35/1	17	3.45	108	13
8911 (s)	15	76	76/0	33**	3.53	94	14
9103 (s)	22	66	57/9	33	2.88	79	18
9105 (s)	22	93	83/10	24	2.95	112	22
8716 (s)	22	110	109/1	28	3.48	87	20
8853 (s)	22	91	63/28	44	3.50	92	21
8668 (b)	33	55	34/21	35	3.70	90	30
8713 (b)	33	96	47/49	75	3.52	102	33
8852 (s,c)	33	95	19/76	65	4.47	126	32
8719 (s,d)	44	94	14/80	38	3.66	119	32
8731 (s)	44	68	17/51	30	3.62	142	32
8851 (s,e)	44	45	5/40	52	3.45	139	32
8857 (s,d)	53	98	15/83	39††	4.15	117	27
8685 (s)	106	46	0/46	38	3.11	89	40
8681 (s,e)	184	54	0/54	37	3.68	126	31
8667 (f)	908	59	0/59	165	4.00	85	34

* Blood pressure rose gradually after nephrectomy.

† Dated from time operation was performed to induce silk-and-turpentine perinephritis.

‡ Early arterial lesions showed necrosis, nuclear pyknosis and/or exudative inflammation of vessel wall. Late arterial lesions showed young, organizing connective tissue or scars in vessel wall.

§ Control levels: Blood urea nitrogen (BUN), 23 ± 4 mg. per 100 ml.; cardiac index, 2.0 to 3.3 × 10^{-3}; blood pressure, 64 ± 5 mm. of Hg.

** Small, necrotic, wedge-shaped infarction in left kidney.

†† Small, recent, hemorrhagic, wedge-shaped infarction in left kidney.

Key: (a) = Death associated with pneumonia.
(b) = Death associated with large hemorrhage(s) in the brain.
(c) = Infarction of right cerebral hemisphere.
(d) = Focal hemorrhages in the brain.
(e) = Hemorrhage in anterior chamber of eye, seen during life.
(f) = Death associated with lung abscess, septicemia and amyloidosis of kidney.
(s) = Sacrificed.

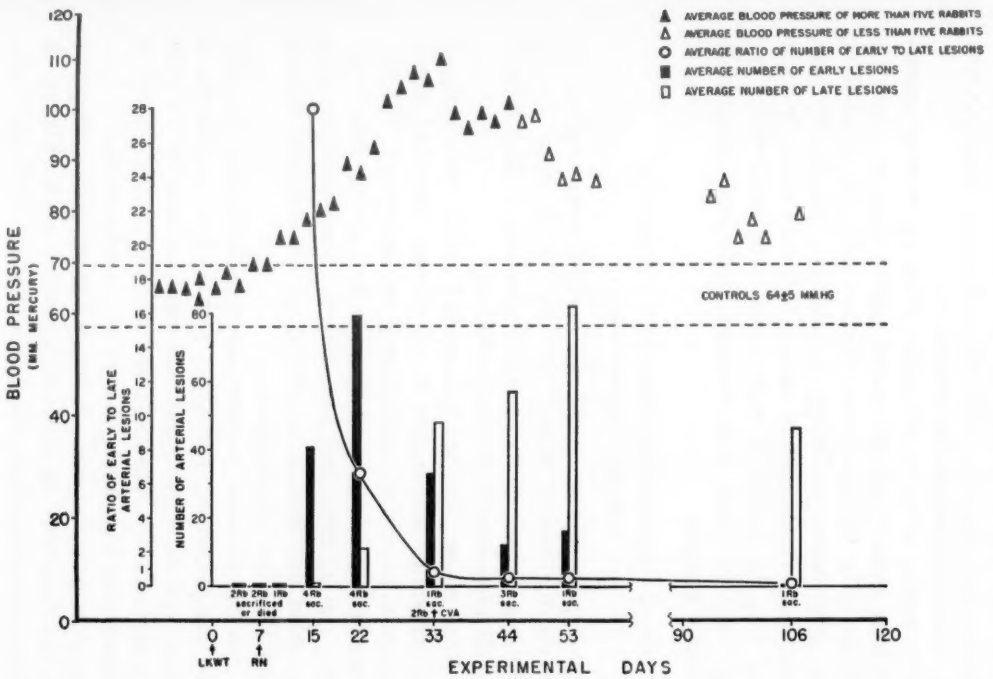
tion. Right nephrectomy was performed 7 days later. Dating from the production of the silk-and-turpentine perinephritis, the rabbits were sacrificed or died as follows: 2 rabbits at 4 days; 2 rabbits at 8 days; 1 rabbit at 10 days; 4 rabbits at 15 days; 4 rabbits at 22 days; 3 rabbits at 33 days; 3 rabbits at 44 days; 1 rabbit at 53 days; 1 rabbit at 106 days; 1 rabbit at 184 days; and 1 rabbit at 908 days. The present report includes 2 animals that had small infarcts in the left kidney, apparently due to tight sutures (Table II). In no other way do these animals differ from the rest with respect to blood pressure elevation, level of blood urea nitrogen, or appearance of arterial lesions.

Blood pressure readings were taken every day or every other day on all animals living up to 53 days; thereafter, these readings were taken on surviving animals every several days or at least once every two weeks.

Results. Results are summarized in Table II. The natural course of the arteritis and hypertension associated with silk-and-turpentine perinephritis is shown in Text-figure 2. The hypertension reached its highest value at 33 ± 6 days after the production of perinephritis. Two rabbits which died at this time were found to have large hemorrhages in the brain at necropsy examination. Thereafter, the blood pressure fell to lower levels, although it continued to be above the expected range of normal throughout the remainder of life in all animals except rabbit 8667. This animal's blood pressure returned to normal 270 days after the initial procedure.

Arterial lesions were present in all rabbits which died or were sacrificed 15 days or more after the production of silk-and-turpentine perinephritis (i.e., 8 days or more after nephrectomy). The microscopic appearance of these lesions is reported elsewhere.[1] The necrotizing arterial lesions were not distinguishable from those occurring in the animals with hypertension and perinephritis produced with dry silk (Figs. 1 to 6), but they were markedly increased in number and were more widely distributed.[1] Between the 22nd and the 53rd experimental days the total number of lesions present on "standard" microscopic examination remained relatively constant. However, the ratio of early lesions to late lesions became inverted, and by the 33rd day late lesions predominated. Early lesions were no longer present 106 days after the production of the silk-and-turpentine perinephritis (Text-fig. 2).

At necropsy the wrapped kidney was surrounded by 2 to 3 cc. of serous exudate in rabbits which died or were sacrificed between the fourth and tenth days after the initial procedure. Fifteen days or more after the first operation, wrapped kidneys were found to be contained



Text-figure 2. Blood pressure after the left kidney was wrapped in silk soaked with turpentine (LKWT), followed in 7 days by right nephrectomy (RN). Widely distributed arterial lesions were not seen until the 15th day after the production of silk-and-turpentine perinephritis and the eighth day after right nephrectomy. By 33 days after the production of perinephritis (26 days after the contralateral nephrectomy), hypertension reached its highest average value, and at this time 2 rabbits died with large hemorrhages in the brain (CVA). On the other hand, the highest average number of early arterial lesions was found 22 days after the production of perinephritis, and late lesions began to predominate by 33 days. Three rabbits sacrificed 106 days or more after the production of perinephritis showed no acute arterial lesions.

in a moderately thick fibrous capsule and were usually paler than normal (Fig. 7).

All perirenal cultures were negative except those of rabbits 8687 and 8686, found dead, 4 and 8 days respectively, after the first operation. These cultures yielded bipolar staining, gram-negative rods. In addition, rabbits 8667 and 8854 died with pulmonary infections, and their blood cultures yielded similar organisms.

The cardiac indices of 13 of 18 rabbits living 15 or more experimental days were greater than the normal control range of 2.0 to 3.3 $\times$ 10^{-3}.[1] Terminally, the blood urea nitrogen values in 12 of these animals were either normal or slightly elevated; in 3 animals they were markedly elevated. In the case of rabbits sacrificed after the 22nd experimental day, the number of early lesions diminished with time, even though terminal blood urea nitrogen levels were appreciably elevated in some animals.

Healed Contralateral Perinephritis Followed by Unilateral Nephrectomy

To test the hypothesis that a rapid rise in blood pressure gives rise to acute necrotizing arterial lesions,[4-6,8-10] and at the same time to elucidate the role of perinephritis in the production of these arterial lesions, the following experiment was performed.

Delayed Nephrectomy. The left kidneys in 8 rabbits were wrapped in silk, and turpentine was applied in the manner described above. Four to 5 months later, right nephrectomy was performed. The animals were sacrificed as follows: 2 rabbits at 16 days after nephrectomy; 2 at 30 days; 2 at 44 days; and 2 at 52 days. Blood pressures were taken every other day prior to nephrectomy and thereafter usually every day until the time of sacrifice.

Results. No rise in blood pressure occurred until after the right nephrectomy was performed. Text-figure 3 shows that a rapid rise in blood pressure occurred within 2 days and continued steeply to a peak 6 days after nephrectomy. The blood pressure then slowly tapered to lower but abnormally high levels 52 days after nephrectomy.

At necropsy no petechiae were observed. The wrapped kidney was pale, small, and constricted by a thin collagenous capsule (Fig. 7). A few histiocytes and foreign body giant cells were present in the thickened perirenal capsule. In the extensive "standard" microscopic examination, no vascular lesions were found.

Cardiac indices were normal in 6 animals and were slightly elevated above the upper limits of normal in 2 rabbits. The level of blood urea nitrogen of 7 animals ranged from normal to slightly elevated values.

The notable exception was rabbit 8690 which had a calculus in the left ureter with hydronephrosis and hydroureter. Terminally, the blood pressure of this rabbit was elevated, and its level of blood urea nitrogen was 277 mg. per 100 ml. The results are summarized in Table III.

TABLE III

*Failure of Development of Arterial Lesions with Healed Unilateral Perinephritis Followed by Contralateral Nephrectomy**

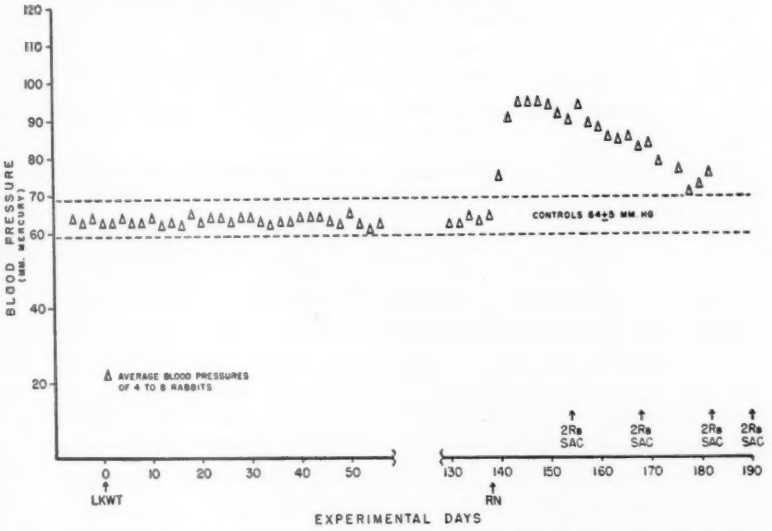
Rabbit no.	Day of sacrifice†	No. of arterial lesions	Terminal BUN‡	Cardiac index‡	Maximum blood pressure‡
8819	16	0	39	2.84	99
8812	16	0	33	2.86	103
8809	30	0	22	2.08	95
8810	30	0	8	2.50	93
8814	44	0	39	2.46	101
8818	44	0	44	2.34	89
8690	52	0	277§	3.36	99
8689	52	0	27	3.38	90

* Blood pressure rose steeply following nephrectomy.

† Dated from time of nephrectomy (see text).

‡ Control levels: Blood urea nitrogen (BUN), 23 ± 4 mg. per 100 ml.; cardiac index, 2.0 to 3.3 × 10^{-3}; blood pressure, 64 ± 5 mm. of Hg.

§ Left ureterolithiasis with hydronephrosis and hydroureter.



Text-figure 3. Blood pressures observed after the left kidney was wrapped in silk (LKWT) followed 4 months later by right nephrectomy (RN). No rise in blood pressure occurred until after nephrectomy was performed. Blood pressures rose within 2 days and continued to rise steeply; the average highest blood pressure occurred 6 days after nephrectomy. The "standard" histologic examination failed to disclose vascular lesions.

Perinephritis with an Intact Contralateral Kidney

Animals with acute or healed unilateral perinephritis and a normal contralateral kidney served as controls for the two previous experiments.

The left kidney of 4 animals was wrapped in silk soaked with turpentine in the manner described previously. After this procedure, the animals were sacrificed as follows: 1 rabbit at 7 days; 1 at 30 days; 1 at 33 days; and 1 at 189 days.

Results. None showed a rise in blood pressure. No vascular lesions were observed in the "standard" microscopic examination in any of the 4 rabbits. The cardiac indices and the levels of blood urea were within normal limits.

Hypertension Induced by Bilateral Carotico-aortic Denervation

Lesions in large arteries, renal arterioles, and glomeruli have been reported in animals with neurogenic hypertension.[11-17] However, there is no evidence that necrotizing arteritis occurs in this condition. To ascertain whether the production of neurogenic hypertension results in necrotizing arteritis, the following experiment was performed.

Two rabbits were used. Carotico-aortic denervation was performed on the right side according to the method of Boyd and McCullagh,[14] and the same procedure was performed 2 weeks later on the left side. Blood pressures were taken every day or every other day throughout the experiment. The animals were sacrificed on the 34th day after the second operation.

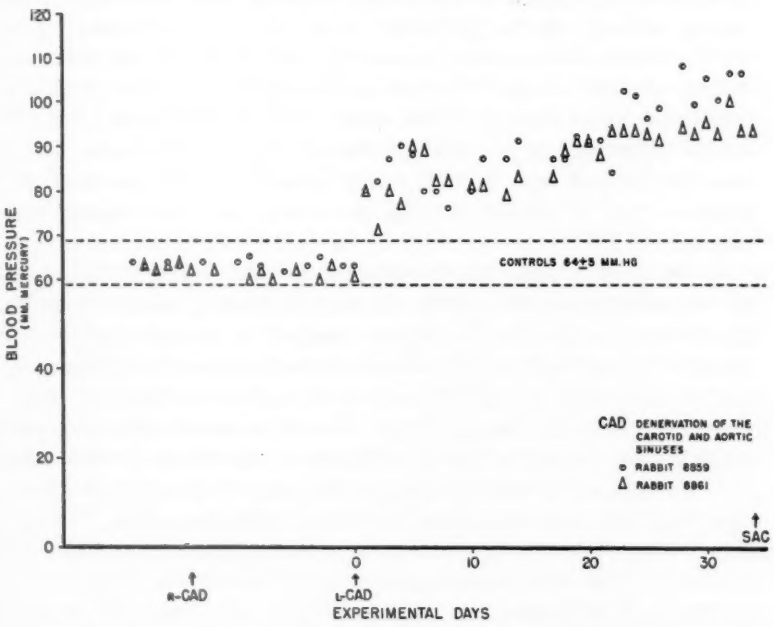
Results. Significant elevation in blood pressure occurred 2 days after the second operation. The blood pressure gradually rose to a plateau at 23 days and persisted at this level until the animals were sacrificed (Text-fig. 4). No vascular lesions were observed in the "standard" microscopic examination. Cardiac indices and levels of blood urea nitrogen were normal.

Controls

Five normal rabbits were used as controls for blood pressure and cardiac indices. Values of blood pressure in these animals were never outside the range of 64 ± 5 mm. of Hg over several months of very many determinations. Their cardiac indices ranged from 2.54 to 2.84 $\times 10^{-3}$. In addition, 9 rabbits which had been sensitized with horse serum also served as controls for cardiac indices; these indices ranged

from 2.39 to 3.18 $\times$ 10^{-3}.[1] Pickering and Prinzmetal[18] found that the cardiac index in 32 normal rabbits ranged from 2.0 to 3.3 $\times$ 10^{-3}.

Three additional rabbits were used to test the effect of a depot of turpentine on blood pressure and arteries in distant tissues. Three cc. of turpentine were dropped over the left kidney of 2 of these ani-



Text-figure 4. Neurogenic hypertension. Right carotico-aortic denervation (R-CAD) and left carotico-aortic denervation (L-CAD) were performed 2 weeks apart in 2 rabbits. The blood pressure rose rapidly immediately after the second procedure and continued to rise until the time of sacrifice. The "standard" histologic examination revealed no vascular lesions.

mals. Seven days later right nephrectomy was performed. These 2 animals were sacrificed 30 days after the first operation. The capsules of the kidneys were slightly thickened, but the cut surfaces were of normal color. In the third animal silk soaked with turpentine was placed between muscles of the posterior abdominal wall. This rabbit was sacrificed 140 days later. Cultures of the inflammation yielded gram-positive cocci. The blood pressure of all 3 of these animals remained within normal limits throughout the experimental periods. None showed vascular lesions in the "standard" microscopic examination. The cardiac indices and levels of blood urea nitrogen were normal.

In control samples of serum from 30 normal rabbits the levels of urea nitrogen were in the mean range of 23 ± 4 mg. per 100 ml.

Discussion

Rabbits were utilized in the experiments here reported because spontaneously occurring necrotizing arteritis had not been found among animals of the particular stock used.[1] Furthermore, other workers have not reported spontaneous necrotizing arterial lesions in rabbits of other stocks.[19,20] In this connection, rats were considered unsuitable by us because of the observations of Wilens and Sproul[21] on the occurrence of necrotizing inflammatory arterial disease in rats over 500 days of age. Indeed, similar arteritis can be provoked with relative ease in young rats by a variety of experimental techniques.[4,5,8,9,22-31]

In the present experiments unilateral aseptic perinephritis induced by wrapping one kidney with silk and performing contralateral nephrectomy 7 days later uniformly resulted in hypertension and in necrotizing lesions of small arteries and arterioles in all rabbits surviving 15 to 53 days. A marked increase in number of identical, widely distributed, vascular lesions (Figs. 1 to 6) occurred when silk perinephritis was intensified by the addition of turpentine (Tables I and II). The animals in which turpentine was used developed higher average blood pressures than those not treated with turpentine. The consistent occurrence of a large number of vascular lesions when turpentine was utilized provided ample material for the correlation of the relative incidence of early and late vascular lesions with the level of blood pressure. The greatest average incidence of early lesions occurred on the 22nd experimental day and antedated the highest average blood pressure by 11 days (Text-fig. 2; Table II). This lack of correlation is well exemplified in the case of rabbit 8716, sacrificed 11 days before the attainment of the highest average blood pressure of the group taken as a whole. This animal exhibited a larger number of arterial lesions in the "standard" histologic examination than other animals in this investigation, and yet its maximum blood pressure was one of the lowest of all those rabbits which developed arteritis. No increase in the average number of acute lesions occurred after the 22nd experimental day, despite the fact that the blood pressure continued to rise to reach an average peak level on the 33rd experimental day; the elevated level of blood pressure persisted for several months thereafter. It is inferred, therefore, that the arterial lesions here designated as "late" represented the end result of an acute process of limited duration.

Although most of the rabbits which developed necrotizing arteritis had slight to moderate elevations of blood urea nitrogen terminally, there was no correlation between this and the occurrence of acute arterial lesions. Moreover, the experimental findings indicated that bacteria or their products played no role in the production of the arteritis.

A direct effect of turpentine on blood vessels can be ruled out as a causative factor in the induction of the acute arterial lesions for the following reasons: (1) The earliest arterial lesions did not appear until 10 to 15 days after the left kidney had been wrapped in turpentine-saturated silk and 4 to 8 days after the right nephrectomy. In fact, the lesions failed to develop if the right kidney was not removed. (2) Lesions were observed neither in the vessels of the wrapped kidney nor in those of the perirenal tissues in direct contact with the turpentine. (3) Neither local nor distant arterial lesions developed when turpentine was placed in the posterior abdominal wall or dropped over one kidney that had not been wrapped in silk. (4) Unilateral silk perinephritis produced without the use of turpentine and followed in 7 days by contralateral nephrectomy resulted in necrotizing arteritis qualitatively identical with that seen in animals in which silk soaked with turpentine was employed (Figs. 1 to 6).

When nephrectomy was performed in the presence of healed rather than acute silk-and-turpentine perinephritis, a more prompt and rapidly rising hypertension resulted than in the case of acute perinephritis (Text-fig. 3). Yet, in this instance, no vascular lesions were observed, although an extensive search was made for them. Furthermore, the prompt and sustained elevation in blood pressure following bilateral carotico-aortic denervation was likewise unassociated with the development of arterial lesions (Text-fig. 4).

These combined observations indicate that sharply rising hypertension did not cause arterial lesions in these rabbits; actually the rise in blood pressure was more gradual in the animals which developed necrotizing arteritis (Text-fig. 2) than in those in which hypertension was not attended by arteritis (Text-figs. 3 and 4). It is inferred, therefore, that the mechanism for the production of hypertension was not identical to that responsible for the production of necrotizing arteritis. In this connection, Koletsky[20] demonstrated that "renal hypertension and vascular necrosis can be dissociated" in adrenalectomized rats with partial infarction of the kidneys.

In our experiments arteritis only occurred when contralateral nephrectomy was performed during the exudative stage of silk perinephritis. In fact, unilateral perinephritis without manipulation of the

contralateral kidney was attended by neither hypertension nor arterial lesions. It would appear, therefore, that a normal kidney served to protect the animals from developing either arteritis or hypertension in the face of acute exudative contralateral perinephritis. If, as the results of these experiments indicate, the mechanism for the production of hypertension and that for the production of necrotizing arteritis appear to be dissimilar, it can be postulated that a circulating substance leading to the production of necrotizing arteritis may be produced during the exudative stage of silk perinephritis. When a normal contralateral kidney is present, this substance would be largely eliminated or inactivated; when, on the other hand, the normal kidney is removed, the retention of the substance could lead to vascular injury. The vessels of the pulmonary circulation and those of the spleen, pancreas, and kidney may not be susceptible to this injury.[1] The amount of such a substance would appear to be directly related to the intensity of the perirenal inflammation since the more intense form of perinephritis caused by turpentine was followed by a larger number of necrotizing vascular lesions when compared to the lesions which followed wrapping the kidney with silk alone.

Summary and Conclusions

Acute necrotizing arterial lesions and hypertension were consistently induced in young rabbits by wrapping one kidney with silk and carrying out contralateral nephrectomy 7 days later. When the same experiment was performed but the perirenal wrapping was soaked with turpentine, a marked increase in the number of arterial lesions and a greater average elevation of blood pressure resulted. Animals sacrificed less than 15 days after production of the silk-and-turpentine perinephritis showed no acute arterial lesions. The largest number of these lesions occurred in animals sacrificed at 22 days, although the elevated blood pressure peak was not reached in 8 of the remaining 10 animals until 30 to 34 days after this procedure. The number of acute lesions fell markedly after 22 days, and late lesions began to predominate at 33 days.

On the other hand, acute necrotizing arterial lesions failed to appear when contralateral nephrectomy was delayed until 4 months after the production of the silk-and-turpentine perinephritis; yet more prompt and rapidly rising hypertension occurred in these rabbits. Moreover, in a supplementary experiment, whereby a rapidly rising neurogenic hypertension was induced, this was not accompanied by the development of acute arteritis.

It appears, thus, that sharply rising hypertension does not cause arterial lesions in rabbits. The existence of a normal kidney protects rabbits from developing either acute necrotizing arterial lesions or hypertension, despite the production of contralateral, exudative, silk perinephritis. It is suggested that a hypothetical circulating substance with the capacity to induce vascular damage is produced during the exudative stage of silk perinephritis. This is largely eliminated or inactivated in the presence of a normal kidney but becomes effective when renal function is reduced by nephrectomy.

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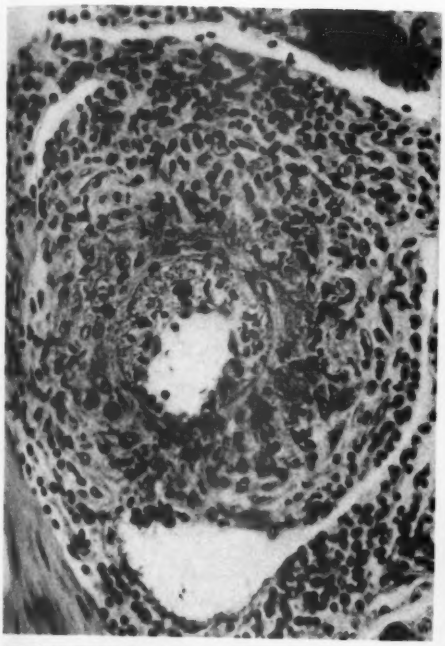
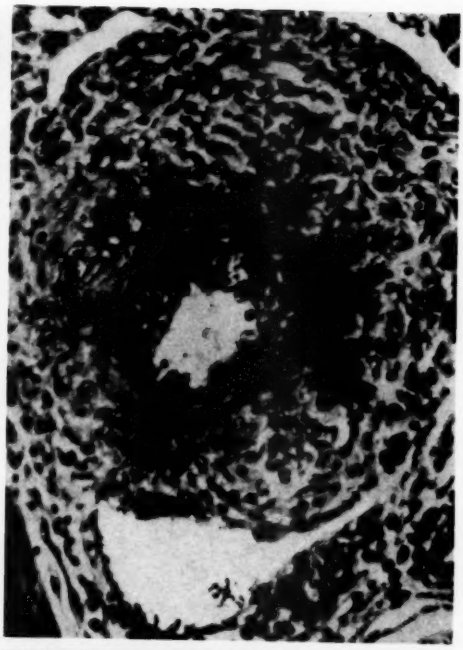
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIGS. 1 and 2. A small artery in the duodenal submucosa of rabbitt 8907 sacrificed 24 days after the left kidney was wrapped in dry silk and 17 days after right nephrectomy. In the intima and media there is extensive circumferential coagulative eosinophilic necrosis (Fig. 1) resembling fibrin in staining character (Fig. 2). Many histiocytes and lymphocytes infiltrate the swollen wall. Figure 1, hematoxylin and eosin stain. × 323. Figure 2, same section restained with phosphotungstic acid-hematoxylin. × 361.

FIGS. 3 and 4. A small artery in the submucosa of the ileum of rabbit 8716 sacrificed 22 days after the left kidney was wrapped in silk soaked with turpentine and 15 days after right nephrectomy. The similarity of this lesion to that shown in Figures 1 and 2 is noteworthy. A few neutrophils and eosinophils are also present. Figure 3, Giemsa stain. × 336. Figure 4, same section restained with phosphotungstic acid-hematoxylin. × 340.





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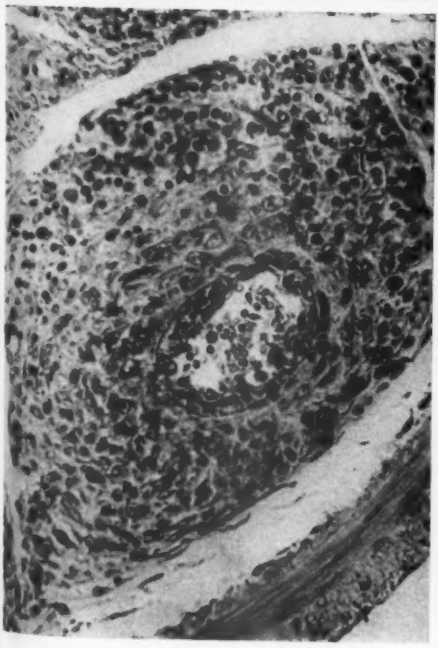

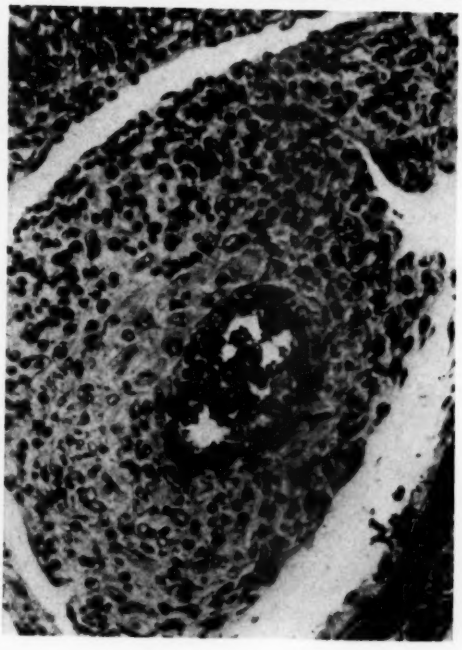

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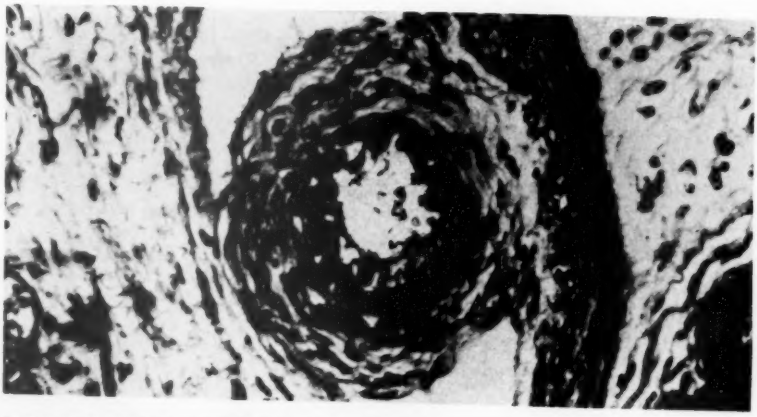
FIG. 5. Small artery bounded by dilated lymph vessels in the submucosa of the cecum of rabbit 8908, sacrificed 35 days after the left kidney was wrapped in dry silk and 28 days after right nephrectomy. Note coagulative necrosis, staining in part like fibrin, in subendothelium and media. Concentric rings of histiocytes, fibroblasts and early collagen are present in the outer adventitia. Phosphotungstic acid-hematoxylin stain. × 362.

FIG. 6. Small artery in the subserosa of the jejunum in rabbit 8713 which died 33 days after the production of the silk-and-turpentine perinephritis. This animal showed multiple petechial hemorrhages in the intestine, kidney, pelvis, ureters, bladder and brain. The similarity of this lesion to that in Figure 5 is noteworthy. Phosphotungstic acid-hematoxylin stain. × 358.

FIG. 7. The formalin-fixed kidneys of rabbit 8679 which had its left kidney wrapped with silk soaked in turpentine 6 months prior to sacrifice. Note that the cortex of the rounder left kidney (A) is pale as contrasted with that of the normal right kidney (B). Kidneys wrapped in silk characteristically have this appearance in the gross.



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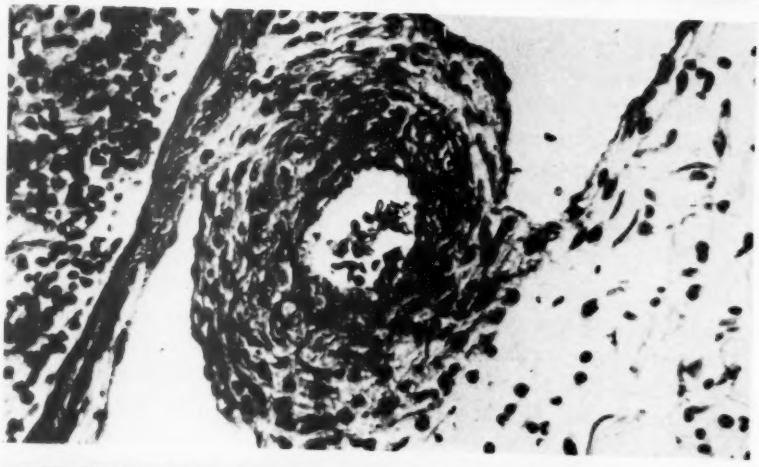

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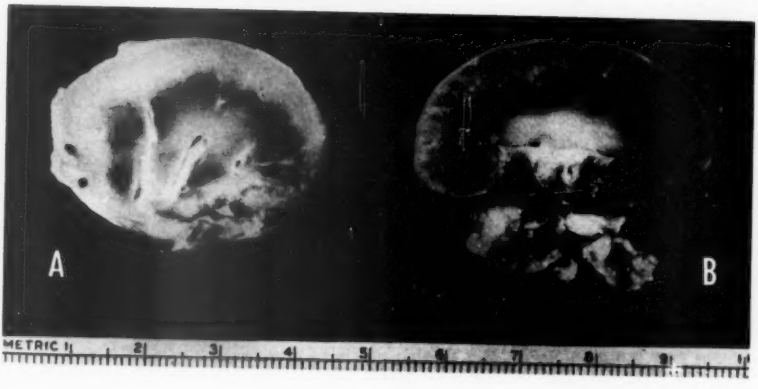

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A STUDY OF THE HISTOCHEMICAL AND STAINING CHARACTERISTICS OF AMYLOID*

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During a study of senile cardiac amyloidosis,[1] it was observed that the manifestation of metachromasia with acidified crystal violet[2] constituted the most reliable indication that suspicious hyaline deposits were indeed amyloid. However, when the staining characteristics of other varieties of amyloid were investigated, it was noted that orthochromatic reactions were frequently encountered in amyloidosis associated with multiple myeloma. This observation prompted a general survey of the staining and histochemical reactions of several varieties of amyloid, the results of which are herein reported.

MATERIAL AND METHODS

Tissues were secured at necropsy from patients dying at the Cincinnati General Hospital and affiliated institutions. Processing by a variety of techniques was unavoidable since a portion of the material had been obtained many years previously. Paraffin blocks from 3 cases of amyloidosis associated with multiple myeloma were furnished us through the kindness of Dr. D. C. Dahlin, Mayo Clinic, Rochester, Minnesota. The manner of preparation and number of cases of each type are indicated at the head of each column in Table I. The techniques applied are indicated in the extreme left-hand column of the chart. Unless otherwise indicated below, the techniques utilized were those in standard use. Sections from cases of senile cardiac, generalized primary, secondary, multiple myeloma and isolated cutaneous amyloidosis were examined. Because of the paucity of material, not all reactions were performed upon all varieties.

Crystal violet staining was performed at concentrations of 0.45 per cent both in dilute hydrochloric acid solution[2] and in buffers at graded pH from 2.0 to 10.0. Hyaluronidases of testicular (Nutritional Biochemicals Corporation, Cleveland, Ohio) and streptococcal (furnished through the courtesy of Wyeth and Company, Philadelphia, Pennsylvania) origin were utilized in digestion studies. The methylation[3] and

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TABLE I

Staining and Histochemical Properties of Amyloid

Reaction	Forms of amyloidosis, manner of preparation, and number of cases				
	Senile cardiac, 5 cases (F/P, F/F)*	Generalized primary, 1 case (Z/P)* Heart, tongue	Secondary, 3 cases (F/P, F/F)* Kidney, spleen	Multiple myeloma, 5 cases: 1 (F/P, F/F), 3 (F/P), 1 (Z/P)* Bone, liver	Isolated cutaneous, 1 case (F/P)*
Crystal violet (0.45%)	Metachromatic pH 2.0 – 9.0	Same	Same	4 of 5 orthochromatic	Metachromatic pH 2.0 – 9.0
Methylation 20 – 25° C.	No change in 72 hr.	Same	Same	No block (1 case)	Same
Methylation 60° C.	Block in 12 hr.	Same	Same	Block (1 case)	Same
Methylation-saponification	Restores metachromasia	Restores metachromasia	Restores metachromasia	Restores metachromasia (1 case)	Restores metachromasia
Acetylation	No block	No block	No block	No block (1 case)	No block
Diastase digestion	No block	No block	No block	No block (1 case)	No block
Pepsin digestion	No block	No block	No block	No block (1 case)	No block
Trypsin digestion	Amyloid dissolved	Same	Same	Same	Same
Hyaluronidase digestion	No effect	Same	Same	Same	Same
Ribonuclease digestion	No effect	Same	Same	Same	Same
Toluidine blue metachromasia					
(a) 3%, 70° C.	+	+	+	4 of 5 orthochromatic	Weak +
After methylation	o	o	o	o (1 case)	o
Methylation-saponification	+	+	+	+ (1 case)	+
(b) 0.25%, room temperature	Weak to absent	Weak to absent	Weak to absent	4 of 5: o 1 of 5: weak to absent	o
After sulfation	Strong	–	Same	+	+
After pepsin digestion	+	–	+	1 of 4 probably +	?
After trypsin digestion	o	o	o	o	–
After pepsin-methylation	o	o	o	o	–
After pepsin-methylation- saponification	Probably +	–	Probably +	? (4 of 5 cases)	–
Diastase PAS	No effect	Same	Same	Same	Same
Pepsin PAS	o	o	o	o	o

After trypsin digestion	o	o	o	o	–
After pepsin-methylation	o	o	o	o	–
After pepsin-methylation- saponification	Probably +	–	Probably +	?	–
Diastase PAS	No effect	Same	Same	Same (4 of 5 cases)	Same
Pepsin PAS	o	o	o	o	o
Bisulfite PAS	Block 2 hr	Same	Same	2 of 5 require 24 hr.	Block 2 hr.
Acetylation PAS	Block 2 hr.	Same	Same	2 of 5 require 24 hr.	Block 2 hr.
Methylation PAS	Block 72 hr. 60° C.	Same	Same	Same	Same
Proteins	+	+	+	+	+
Naphthol Y SX	+	–	+	–	–
Millon	+	+	+	+	+
Ninhydrin-Schiff†	Weak +	Same	Same	Same	Same
Coupled tetrazonium	+	+	+	+	+
Post PTA-aniline blue (Masson, Mallory-Heidenhain)	Blue	Blue	Blue	2 blue; 2 red; 1 mixture	Blue
Phosphotungstic acid-hematoxylin	Pink	Pink	Pink	3 of 5 blue	Pink
Acid fuchsin (van Gieson)	o	o	o	o	o
Feulgen	o	o	o	o	o
Lipids					
Oil red O	o	o	o	o (1 case)	o
Luxol fast blue	o	o	o	o	o
Sudan black B	o	o	o	o	o
Baker's acid hematein	o (1 case)	–	–	o (1 case)	–
Miscellaneous					
Alcian blue	Usually +	+	Usually o	o	Weak +
Alcian green	Usually +	+	Usually o	o	Weak +
Alcian blue-PAS	Mixed	Mixed	Usually red	Usually red	Mixed

* F/P = Formalin fixation, paraffin embedded.
F/F = Formalin fixation, frozen sections.
Z/P = Zenker fixation, paraffin embedded.
† Only formalin-fixed tissue available.

o = Negative reaction.
+ = Positive reaction.
? = Reaction indeterminate because of poor preservation.
– = Reaction not performed.

saponification[4] techniques were those described by Lillie. When these rigorous techniques followed peptic digestion, survival of the tissue posed a considerable problem, but the use of the concentrated toluidine blue technique described below provided confirmation of the findings to be described. Sulfation was accomplished by means of the technique described by Moore and Schoenberg.[5] Toluidine blue was used both in buffered dilute (0.25 per cent) solution at room temperature and in concentrated (3 per cent) solution, buffered to pH 2.9 at 70° C.[6]

Results

The results are indicated in Table I. The salient features are summarized as follows:

1. In all varieties of amyloid, protein content was prominent.
2. Amyloid in multiple myeloma, in contrast to other varieties tested, usually was orthochromatic with crystal violet.
3. Other varieties of amyloid manifested strong metachromasia with crystal violet or with 3 per cent toluidine blue at 70° C., but weak to absent metachromasia with dilute (0.25 per cent) toluidine blue at room temperature.
4. Sulfation or pepsin digestion resulted in the production of metachromasia with toluidine blue; methylation blocked metachromasia with crystal violet. Methylation also blocked toluidine blue metachromasia produced by peptic digestion or the use of 3 per cent toluidine blue concentration at 70° C.
5. When methylation was followed by saponification with potassium hydroxide, metachromasia was restored.
6. The periodic acid-Schiff (PAS) reaction was positive in all cases; frequently it was stronger in secondary amyloid and amyloid with myeloma.
7. Two-hour bisulfite treatment or acetylation, or peptic digestion all blocked the PAS reaction. There was, however, greater resistance to the blocking by bisulfite and acetylation in some cases of amyloid with myeloma.
8. Phosphomolybdic acid-phosphotungstic acid-aniline blue and phosphotungstic acid-hematoxylin (PTAH) methods produced reactions resembling those of collagen in all varieties of amyloid except in that associated with multiple myeloma; all reacted with acid fuchsin (van Gieson) stain in a fashion unlike collagen.
9. Lipid was absent.

Discussion

Many definite and certain speculative interpretations of these results are warranted. It seems reasonable to conclude that most amyloids contain two carbohydrate components manifesting independent histochemical characteristics. One of these is a PAS-positive component (probably glycoprotein[7]), blocked by acetylation and digested by pepsin. The other is an acid mucopolysaccharide which yields metachromasia with crystal violet but not with dilute toluidine blue. This metachromasia is neither blocked by acetylation nor digested by pepsin. The observation that metachromasia with toluidine blue is revealed after peptic digestion has been interpreted by Windrum and Kramer[8] to indicate that anionic dye binding sites are blocked in the amyloid moiety by combination with glycoprotein.

Larsen[6] demonstrated metachromasia of amyloid with toluidine blue when acidified concentrated solutions (3 per cent) and high temperatures (70° C.) were used. He also showed that the presence of free proteins interfered with the demonstration of metachromasia of acid mucopolysaccharides when dilute solutions of toluidine blue were utilized. He postulated that there was a competition between proteins and toluidine blue molecules for the anionic dye binding sites of acid mucopolysaccharides. At high temperatures (70°) and low pH (2.9), highly concentrated dye cations could exchange with the blocking proteins and metachromasia thus become manifest. Confirmatory evidence indicating the presence of acid mucopolysaccharides was provided by the usually positive reactions with both Alcian blue and Alcian green.

The data herein described would further tend to indicate that the acid polysaccharide was carboxylated rather than sulfated or phosphorylated. As pointed out by Kantor and Schubert,[9] methylation of acid mucopolysaccharides eliminates their metachromasia, but by different mechanisms in the case of sulfated, as opposed to phosphorylated or carboxylated compounds. In sulfated polysaccharides, the sulfate group is removed by means of methylation, resulting in the substitution of a hydroxyl group and the formation of free methyl sulfate esters. With carboxyl or phosphoryl groups, the acidic groups remain attached and become esterified. In these latter cases, saponification restores the original structure[4,10,11] and, therefore, the property of metachromasia, whereas with sulfated mucopolysaccharides, restoration of the property of metachromasia by saponification is im-

possible, since the sulfate groups are no longer attached to the polysaccharide molecules.

For sulfated acid mucopolysaccharides:

$$R - OSO_3K + CH_3OH \xrightarrow{HCl - 60^\circ} ROH + CH_3OSO_3K$$

For carboxylated acid mucopolysaccharides:

$$R - COOH + CH_3OH \xrightarrow{HCl - 60^\circ} RCOOCH_3$$

$$R - COOCH_3 + KOH \xrightarrow{70\% \text{ alcholic}} R - COOR + CH_3OH$$

This sequential procedure causes the restitution of crystal violet metachromasia and the metachromasia with toluidine blue following peptic digestion or the use of 3 per cent toluidine blue at 70°. These findings appear to preclude the possibility that a sulfated compound may account for the metachromasia of amyloid. The possibility that the metachromasia is due to phosphorylated nucleic acid appears to be eliminated by the lack of effect of ribonuclease digestion on metachromasia and a negative Feulgen reaction.

The data concerning the character of the protein component of amyloid yielded by this survey are not specific. Although tests for various amino-acid components are positive, these results offer no definite indication of the nature of the protein. In particular, they do not demonstrate the presence of serum globulins.

The usually positive reaction of amyloid with aniline blue following treatment with phosphotungstate or phosphomolybdate in the Mallory-Heidenhain or the trichrome staining techniques is noteworthy. According to Puchtler and Isler,[12] this quality indicates a high content of basic groups, the phosphomolybdic acid (PMA) forming a bridge between the basic substrate and the alkaline component of the amphoteric dye. The more basic proteins (collagen, reticulin, and presumably amyloid) are thus stained selectively by the aniline blue or other similar dye. The smaller number of such groups contained in less basic proteins (cytoplasm, sarcoplasm, etc.) bind only a relatively small amount of the PMA, resulting in their staining minimally with such dyes. These latter components may be stained with an acid dye before treatment with the phosphotungstic acid (PTA) or PMA.

Since amyloid is usually indistinguishable from collagen by the PTA-PMA-aniline blue or trichrome techniques, it is necessary to make this distinction with the van Gieson method. The failure of amyloid to stain with acid fuchsin by this means might be interpreted to indicate a less basic protein than that of collagen.

The apparently unique behavior of the amyloid seen in 4 of 5 cases

of multiple myeloma is noteworthy and would appear to indicate a difference in composition. Most cases revealed no metachromasia with crystal violet, nor was there metachromasia with toluidine blue following peptic digestion or with 3 per cent concentration at 70° C. The PAS reaction usually was stronger in amyloid with multiple myeloma, and, in some cases, was more resistant to blocking by bisulfite treatment and acetylation. One might speculate that a greater proportion of glycoprotein and protein causes a more profound inhibition of the metachromasia of any acid mucopolysaccharide present, or, as seems more likely, that the acid mucopolysaccharide component is frequently lacking in the "amyloid" seen in multiple myeloma. The variability of other tinctorial reactions of amyloid in myeloma contrasted with other forms further supports this latter hypothesis and suggests variation in chemical composition from case to case. Indeed, amyloid in multiple myeloma is unique histologically in that it may be encountered intracellularly within neoplastic plasma cells,[13] here resembling the hyaline material of Russell bodies.[14] The single case of 5, which manifested metachromasia with crystal violet, also was metachromatic with 3 per cent toluidine blue at 70° C. The acid polysaccharide in this case appeared histochemically similar to that of other amyloids.

Furthermore, the characteristics of the protein component of amyloid in myeloma are essentially different from those noted in other varieties. The reaction with the PTAH and aniline blue techniques, as in Mallory-Heidenhain's or Masson's stains is frequently (3 of 5 cases) unlike that of connective tissue. On the other hand, other varieties showed tinctorial qualities resembling those of collagen with these stains. This behavior would suggest that the protein in these cases differs in nature from that seen in other forms of amyloid, probably being of a less basic character. It would tend to confirm the presence of variation in chemical composition. One cannot avoid speculating that this type of "amyloid" may represent a simple precipitate of abnormal proteins (globulins ?) or glycoproteins.

Although there were slight differences observed in the reactions of secondary amyloid from those of the primary form in our material, these were relative rather than absolute. While the PAS reaction generally appeared somewhat stronger in the secondary variety, it was blocked as readily as that in the primary type. While there was usually a negative reaction with Alcian blue and no metachromasia with 0.25 per cent toluidine blue, metachromasia of secondary amyloid after pepsin digestion or when stained with 3 per cent toluidine blue at 70° C. did not differ from that of primary amyloid. The limitation of material precluded the possibility of a complete survey of isolated cutaneous

amyloidosis, but the several reactions described were similar to those of the primary cardiac variety.

A brief comparison with other reports of this nature is in order. From the standpoint of metachromasia with toluidine blue, our data generally agree with those of Windrum and Kramer,[8] and Larsen[6] rather than those of Wagner,[15,16] who described amyloid as metachromatic when stained with dilute toluidine blue solutions. Our observations on the effects of peptic digestion and the use of concentrated solutions of toluidine blue at high temperatures also confirm those of Windrum and Kramer[8] and Larsen[6] and extend them to the primary variety.

The suggestion that the acid polysaccharide component is carboxylated rather than sulfated is at variance with the conclusions reached by Hass,[17] who believed that it was chondroitin sulfate. Ehrström[18] observed that a mixture of serum and chondroitin sulfate manifested similar tinctorial properties to those of amyloid. Meyer,[19] on the other hand, suggested that the acid polysaccharide present was monosulfated and related to heparin. More recently, Giles and Calkins[20] reported that chondroitin sulfate was not a prominent component of secondary amyloid. They found, on chemical analysis, that it contained protein and no more than 4 per cent carbohydrate, with both glycoprotein and polysaccharide contributing to the latter component. Wagner[15] found mucopolysaccharide and globulin in amyloid on chemical analysis. Larsen[7] pointed out the prominence of the glycoprotein component. Diametrically opposed to all these observations is the study of Eppinger[21] which revealed no polysaccharide component.

Histochemical study offers the advantage of certain knowledge of the tissue component that is yielding the reaction utilized. It is possible that the variability in chemical analytic data is the result of the existence in tissue extracts of other carbohydrate tissue components (glycoprotein, chondroitin sulfate) universally present in all stromal tissues.[22] The present study appears to offer strong evidence that a non-sulfated acid mucopolysaccharide and a glycoprotein are present in amyloid.

The data of Vazquez and Dixon[23,24] and of Wagner,[25,26] using fluorescent antibody techniques and chemical analysis respectively, tend to indicate that globulin is an important component of amyloid. These investigators have suggested that this substance represents an antigen-antibody precipitate. Calkins, Cohen, and Gitlin[27] have secured data using immunochemical methods tending to deny this hypothesis. The ordinary histochemical techniques may not be expected

to resolve this problem, but the data secured in this study appear to constitute evidence against the hypothesis that amyloid represents a simple antigen-antibody precipitate.

Summary and Conclusions

1. A study was made of the staining and histochemical reactions of 5 different varieties of amyloid.

2. The results tended to indicate that metachromasia with crystal violet, and with toluidine blue after peptic digestion, or in 3 per cent concentration at 70° C. is due to the presence of non-sulfated acid mucopolysaccharide, probably of carboxylated nature.

3. Three components are identifiable—protein, carbohydrate (or glycoprotein), and acid mucopolysaccharide.

4. "Amyloid" associated with myeloma was variable in tinctorial reactions, usually not metachromatic, and its protein and carbohydrate components frequently differed, histochemically, from those of other varieties of amyloid. It is suggested that this variety of "amyloid" may frequently possess little or no acid mucopolysaccharide, and perhaps less basic protein than other varieties.

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HYALINIZATION OF THE ISLETS OF LANGERHANS IN NONDIABETIC INDIVIDUALS*

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For many years following Opie's discovery of hyalinized pancreatic islets in diabetic patients,[1,2] this lesion was considered diagnostic of diabetes mellitus. But during the intervening years, there have been several reports of hyaline islets in subjects without clinical evidence of diabetes. Arey[3] found hyaline islets in 19 of 114 nondiabetic persons over 50 years of age. Hartroft[4] stated that the amount of hyaline in the islets increased with age in both diabetic and nondiabetic individuals; but at any given age the extent and severity of hyalinization was greater in those having diabetes than in those without it. However, no extensive studies have been reported on the incidence of hyaline islets in nondiabetic individuals.

In 1952 I reported the frequency and age distribution of hyaline islets in 1,194 diabetic patients.[5] The present study includes 1,661 such patients (Tables I and II). It will be noted that the frequency of hyaline islets in these cases increased with age until about the seventh decade, and that there was no significant difference with respect to sex.

The purpose of this study is to determine the frequency and the age and sex distribution of hyaline islets in the nondiabetic population. It is based upon microscopic examination of the pancreas in 3,959 nondiabetic individuals over 20 years of age. There were 2,413 males and 1,546 females. The age distribution is shown in Tables I and II. It will be noted that 1,906 males and 1,143 females were over 50 years of age. The observations were made on one or two sections of pancreas each, usually about one square centimeter in area. The number of islets seen ranged from 10 to 100 or more, depending upon the part of the pancreas from which the section was taken. Since often only one hyaline islet was observed in a section, it is highly probable that a more extensive study would have revealed a larger number of cases with this feature, but this is a comparison of a diabetic with a nondiabetic population, and the pancreases examined in diabetes are subject to the same error of underestimation.

In Tables I and II the incidence and degree of hyalinization of the islets in diabetes and in the nondiabetic state are compared. The first

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two decades have been omitted since no hyaline islets were found in either group under the age of 20 years. In the diabetic group the incidence of hyaline islets increased with age and attained a maximum at about the age of 60 years; in the nondiabetic patients the incidence continued to increase with advancing age to reach a maximum in the ninth decade. In the nondiabetic individuals the proportion of those with mild or moderate hyalinization (grades 1 and 2) was much

TABLE I

The Incidence of Hyaline Islets in Diabetic and Nondiabetic Males with Respect to Age

Age at death		No. of cases	Extent of hyalinization		Percentage with evidence of hyalinization
			Grades 1 and 2	Grades 3 and 4	
20–30	D	16	1	0	6.3
	ND	101	0	0	0
30–40	D	50	2	0	4.0
	ND	181	1	0	0.6
40–50	D	64	8	8	25.0
	ND	225	2	1	1.3
50–60	D	136	31	21	38.2
	ND	367	12	7	5.2
60–70	D	271	74	46	44.3
	ND	727	64	7	9.8
70–80	D	195	66	40	54.4
	ND	507	61	11	14.2
80–100	D	66	19	16	53.0
	ND	305	45	10	18.0

D = diabetic.
ND = nondiabetic.

TABLE II

The Incidence of Hyaline Islets in Diabetic and Nondiabetic Females with Respect to Age

Age at death		No. of cases	Extent of hyalinization		Percentage with evidence of hyalinisation
			Grades 1 and 2	Grades 3 and 4	
20–30	D	32	1	0	3.1
	ND	129	0	0	0
30–40	D	38	4	0	10.5
	ND	124	0	0	0
40–50	D	64	12	3	23.4
	ND	150	1	0	0.7
50–60	D	149	37	25	41.6
	ND	168	8	0	4.8
60–70	D	282	86	49	47.9
	ND	371	21	1	5.9
70–80	D	225	74	39	50.2
	ND	377	40	4	11.7
80–100	D	73	26	12	52.1
	ND	227	29	4	14.5

D = diabetic.
ND = nondiabetic.

TABLE III

The Incidence of Diabetes in the Necropsy Population with Respect to Sex and Age at Death

Age at death (yrs.)	Males			Females		
	No. of necropsies	No. of diabetics	Per cent diabetic	No. of necropsies	No. of diabetics	Per cent diabetic
0–10	7344	9	0.12	5206	2	0.04
10–20	1222	10	0.82	1004	16	1.59
20–30	2211	23	1.04	1889	40	2.12
30–40	3531	69	1.95	2318	53	2.29
40–50	5943	86	1.45	3117	77	2.47
50–60	9211	193	2.10	4039	209	5.17
60–70	10534	360	3.42	4750	361	7.60
70–80	7780	250	3.21	4298	285	6.63
80–93	3122	82	2.65	2145	92	4.29
Total	50898	1082		28766	1135	

TABLE IV

Number of Subjects with Hyaline Islets per Each 1,000 Necropsies with Respect to Age and Sex. (Constructed from Data in Tables I, II and III)

Age at death	Diabetic patients		Nondiabetic patients		Total hyaline islets per 1,000 necropsies	Per cent of hyaline islets attributable to diabetes
	No.	No. with hyaline islets	No.	No. with hyaline islets		
Males						
50–60	21	8	979	51	59	13.6
60–70	34	15	966	95	110	13.6
70–80	32	17	968	137	154	11.0
80–93	26	14	974	175	189	7.4
Females						
50–60	52	22	948	46	68	32.4
60–70	76	36	924	55	91	39.6
70–80	66	33	934	109	142	23.2
80–93	43	22	957	139	161	13.7

greater than in the cases with diabetes, but some nondiabetic patients, especially males, showed severe hyalinization (Table I).

In all decades and in both sexes, hyaline islets were found much more frequently in diabetes than in its absence. In males (Table I) the preponderance was 18 to 1 in the fifth decade but decreased to 3 to 1 in the ninth. In females (Table II) the preponderance in diabetes was even more pronounced since hyaline islets were less frequent in nondiabetic females than in nondiabetic males.

The preponderant occurrence of hyaline islets in diabetic patients is so pronounced that no one can doubt that they are related in some

way to the diabetic state. It is obvious, however, that the observation of hyaline islets at necropsy does not justify a diagnosis of diabetes mellitus.

Table IV is constructed from the data provided in Tables I, II and III. Column 1 is the number of diabetic individuals per each 1,000 necropsies (10 times the percentage in Table III). Column 2 is the number of diabetic patients in whom hyaline islets were encountered (number of diabetic cases times the percentage with hyaline islets). Column 3 is the number of nondiabetic persons per 1,000 necropsies (1,000 less the number with diabetes). Column 4 is obtained by multiplying the number of nondiabetic patients by the percentage of those whose pancreases contained hyaline islets (Tables I and II). When the data are arranged in this way, one may readily determine the percentage of hyaline islets attributable to diabetes in each sex and age group. For example, in males 70 to 80 years old there was only one chance in 9 that a hyaline islet represented clinically detectable diabetes; but in a female 60 to 70 years old there were 2 chances out of 5 that diabetes existed.

The Significance of Hyaline Islets

As noted above, hyaline islets must have some relation to the diabetic state. They are evidently not the cause of diabetes since they were absent in nearly all young persons with diabetes and about one half of older diabetic individuals. In diabetes the hyaline islets are not related to any feature of the disease except the age of the patient.[5] Two theories of their significance may be discussed:

(1) Hyaline islets represent a wear-and-tear process, due to age, which is accentuated by the diabetic state. We know that diabetes does not cause atherosclerosis, but it accelerates and intensifies certain forms of atherosclerosis, viz., renal vascular disease, gangrene, and coronary artery disease. This theory would explain the very high incidence of hyaline islets in nondiabetic individuals over 80 years of age, but it does not account for the greater incidence of hyaline islets in males in the nondiabetic group.

When the nondiabetic patients were arranged in several groups such as those with atherosclerosis, cancer, infections, or trauma, no differences in the incidence of hyaline islets in the several groups were noted except those assignable to age. There was no relation to atherosclerosis. Pancreases which were the seat of atherosclerosis showed no increased incidence of hyaline islets.

(2) Hyaline islets are an expression of unrecognized or potential

diabetes. Since the inheritance of diabetes is not sex-linked, one would expect to find an equal sex distribution; but in the community from which these cases stemmed, clinical diabetes is about twice as frequent in females as in males. This fact justifies the view that unrecognized or potential diabetes is about as frequent as overt diabetes in males, thereby accounting for the higher incidence of hyaline islets in nondiabetic males since more male diabetic patients are unrecognized. Hyaline islets are just as frequent in mild as in severe diabetes.[5] The theory that hyaline islets indicate potential diabetes does not explain their very high incidence in subjects over 80 years of age.

Steinberg and Wilder[6] suggested that the total diabetic population, including undiagnosed and potential diabetic patients, was 5 times as great as the number of recognized instances of diabetes. If this be true, nondiabetic persons with hyaline islets may represent individuals with undiagnosed or potential diabetes.

Summary

In males the incidence of pancreatic hyaline islets in diabetes, in respect to a necropsy population without diabetes, ranges from 18 to 1 in the fifth to 3 to 1 in the ninth decade. In females the preponderance in diabetic individuals is even more pronounced. There is no doubt that hyaline islets are related in some way to the diabetic state.

When hyaline islets are found at necropsy in a male over 50 years of age, the chance is only about 1 in 10 that the patient was recognized to have diabetes in life. In females of corresponding age, the chance is about 1 in 4.

There is some evidence to support the view that nondiabetic individuals with hyaline islets are potential or unrecognized instances of diabetes.

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HISTOCHEMICAL DETECTION OF FATAL ANTICHOLINESTERASE POISONING

II. Reactivation of Cholinesterase in Cadavers of Rats *

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In 1951 it was shown[1] that cholinesterase (ChE) was stable in postmortem muscle and was demonstrable by the Koelle technique[2] which served to localize the enzyme histochemically. It was noted then that, following death resulting from the action of some of the anticholinesterases, there was greater deposition of copper sulfide (CuS) in muscle when necropsy was delayed for 24 hours than when it was performed immediately after death. Since ChE activity was assessed by the amount of CuS precipitate, one could infer that some spontaneous reactivation of enzyme might have occurred during the interval between death and a delayed necropsy.

In cases of suspected poisoning by anticholinesterases, the possibility of spontaneous reactivation of ChE in cadavers has been a factor to reckon with when there was a delay in transit or when tissue was sent for testing from great distances. In such instances, the estimate of ChE inhibition probably did not accurately indicate the amount of inhibition at the time of death. Consequently, the usefulness of the technique for medicolegal purposes was limited. It was decided, therefore, to examine muscle at intervals after death from animals sacrificed by the administration of several anticholinesterases which were known to differ among themselves in reversibility in order to learn whether spontaneous reactivation could be detected histochemically.

An additional limitation to the usefulness of this technique was communicated by Petty[3] who informed the author that in legal medicine, inability to demonstrate ChE histologically is not acceptable as adequate proof of anticholinesterase poisoning. Obviously, the value of such a finding would be enhanced if the site of the enzyme, although inhibited, could be demonstrated by another test. Since the ability of oximes to reactivate ChE after inactivation by various anticholinesterases, both *in vivo*[4,5] and *in vitro*[6-13] is well known, the use of an oxime for such a second test was investigated. Samples which in the first series of tests had shown the greatest amount of inhibition and the least spontaneous reactivation were used.

* Received for publication, December 8, 1958.

Experimental Procedure

Male albino rats weighing approximately 200 gm. received intraperitoneal injections of the following compounds, all of which have an anticholinesterase action: Diazinon [o-o-diethyl-o-(2-isopropyl-4-methyl-pyrimidyl (6)) thiophosphate], Parathion (o,o-diethyl-o-p-nitrophenyl thiophosphate), TEPP (tetraethyl-pyrophosphate), Sarin (isopropylmethylphosphonofluoridate), Tabun (ethyl dimethylphosphoroamidocyanidate), and DFP (diisopropyl phosphorofluoridate). In the tables the number of injections is given in parentheses. The animals were kept at room temperature (24° to 29° C.) for 24 hours after death. Samples of striated muscle were taken from the same animal within 5 minutes after death and at 2 and 24 hours after death; in the case of TEPP and Parathion, samples were taken at 2 and 24 hours only. Tissue was kept in dry ice until the histochemical procedure was carried out. Control muscle was obtained from animals sacrified by inhalation of open drop ether.

Immediately upon thawing, a muscle sample was teased apart in saline solution so that each fragment consisted of approximately 50 or fewer fibers. For a study of the spontaneous reversal of anticholinesterase inhibition, 6 to 8 of these muscle fragments were placed immediately in the incubation solution with acetylthiocholine as substrate. This solution had been made according to Koelle's 1951 formula.[2] The tissue was incubated for 15 minutes at 37° C. and subsequently mounted on a slide in glycerin. The cover slip was sealed with mastic-paraffin. The subneural apparatus of the motor end-plate, the site of ChE activity, was made visible by a copper sulfide (CuS) precipitate. Usually several hundred motor end-plates were seen in the pieces of muscle under the cover slip. The degree of ChE activity was assessed by the amount and intensity of the precipitate, ++++ denoting maximum activity and o denoting no activity detectable by this technique. Grades in between these limits were designated, in descending order, as +++, ++, + and ± (where only a trace of CuS was visible). When the intensity of the precipitate varied within a sample, this variability was indicated by the listing of the two or more grades, the most common being listed first. Grades in parentheses apply to only a few motor end-plates.

The oximes used were 2-PAM (pyridine-2-aldoxime methiodide) and TMB4 (1, 1′-trimethylene bis [4-formylpyridinium bromide] dioxime). They were used in a concentration of 1×10^{-3} M in saline. Teased muscle was immersed for 30 minutes at room temperature (24° to 29° C.) in the solution containing the oxime, then rinsed for approxi-

mately 1 minute in saline and immediately afterwards placed in incubation solution for the Koelle test. In the series of tests performed to show the reactivating effect of 2-PAM, the tests were run in pairs, one portion of the teased muscle being kept frozen while the other was immersed in the 2-PAM saline solution. In the series performed to compare the reactivating effect of TMB_4 with that of 2-PAM, a sample of muscle was teased and divided into 3 portions. One portion was immersed in saline while the second was immersed in TMB_4 saline solution and the third in 2-PAM saline solution.

Results

Spontaneous Reversal of Anticholinesterase Inhibition in Rat Cadavers

The data of this experiment are recorded in Table I. Following death from the insecticides Diazinon and Parathion, there was considerable deposition of CuS in muscle that had not been removed from the animal until 24 hours after death. There was much less in muscle removed from the same animal within 5 minutes or at 2 hours after death. These results indicated considerable spontaneous reactivation of the inactivated enzyme in the animal during the 22 hour interval. Following death from TEPP and Sarin the same alteration could be detected, but the differences were less marked. In the samples taken at 5 minutes and 2 hours after death, there was an appreciable although variable amount of CuS precipitate. After poisoning with Tabun, the CuS deposition was reduced in samples taken 24 hours after death, and it did not differ, with one exception, from the amount seen in the 5 minute and 2 hour samples. Apparently not much spontaneous reactivation occurred in the dead animal during the 24 hour period. After poisoning with DFP, no CuS was deposited (traces in 2 instances), and no spontaneous reactivation occurred during the 24 hour period after death.

Reversal of Anticholinesterase Inhibition by Pyridine-2-aldoxine methiodide (2-PAM)

The data of this experiment are recorded in Table II. The samples selected for this series were those which had shown marked inhibition of CuS deposition in the previous experiment (Table I) and which had been stored in dry ice in the interim. The reactivating effect of 2-PAM on ChE inhibited by Diazinon, Parathion and Sarin is shown by the greater amount of CuS precipitate. In general, it was equivalent to

TABLE I

Spontaneous Reversal of Anticholinesterase Inhibition in Rat Cadavers Held at Room Temperature (24° to 29° C.)

Method of sacrifice	Animal no.	Total dose (mg. per kg.)	Interval from first exposure to death	Kind of muscle	CuS deposit at motor end-plates in muscle taken at intervals after death of animal		
					After 5 min.	After 2 hr.	After 24 hr.
Diazinon*	1	200 (1)†	13 min.	Intercostal	+		+++
				Gastrocnemius	+	+	
				Triceps			+++
	2	200 (1)	13 min.	Intercostal			+++
				Gastrocnemius	+, ±	+	
				Triceps			++, +++
Parathion	1	127 (1)	15 min.	Intercostal			+++
				Gastrocnemius		+	++, +++
	2	127 (2)	150 min.	Intercostal			+++
				Gastrocnemius		+	+++, ++++
TEPP	1	1.1 (1)	10 min.	Intercostal			+++, ++++
				Gastrocnemius		++, (+++)‡	
Sarin	1	0.225 (2)	30 min.	Intercostal		±, +	++++, +++
				Gastrocnemius	++		++
				Triceps		+++	
	2	0.225 (2)	50 min.	Intercostal		+++	+++, ++++
				Gastrocnemius	+, ++		+, (++)
				Triceps		+	
Tabun	1	1.25 (1)	3 min.	Intercostal		++, +	++, +
				Gastrocnemius	+, ++, o		o
				Triceps		o, (+)	
	2	1.25 (3)	90 min.	Intercostal		o	±
				Gastrocnemius	o		±, o
				Triceps		o	
	3	1.00 (2)	61 min.	Intercostal		+, ±, o	+++, ++
				Gastrocnemius	o, (+)		+, o, (±)
				Triceps		+	
DFP	1	5.0 (2)	13 min.	Intercostal		o	o
				Gastrocnemius	o		o
				Triceps		o	
	2	5.0 (2)	14 min.	Intercostal		o	o, (±)
				Gastrocnemius	o, ±		o
				Triceps		o	

* For chemical composition of the anticholinesterases, see text (Experimental Procedure).
† Number of injections indicated by figure in parentheses.
‡ Grade in parentheses applies to only a few motor end-plates.

the spontaneous recovery seen in animals 24 hours after death. Use of 2-PAM on the DFP-poisoned muscle effected only slight deposition of CuS, but it was sufficient to reveal sites of the motor end-plates and to furnish proof, therefore, that motor end-plates were included in the sample of muscle.

Reversal of Anticholinesterase Inhibition by 1, 1'-trimethylene bis [4-formylpyridinium bromide] dioxime (TMB4)

The reactivation of inhibited ChE by an equimolar amount of TMB4 was determined in order to compare its effectiveness with that of 2-PAM. In this experiment, as indicated in Table III, one portion of tissue in each series was immersed in saline only while the other two were immersed in either TMB4 saline or 2-PAM saline. The sampling was limited to muscle from animals poisoned by Tabun, Sarin and DFP, that is, to muscle in which the least spontaneous reactivation had been found previously. Only tissues poisoned by Sarin revealed a slight reactivating effect in saline alone. The greater effectiveness of TMB4 as compared with 2-PAM is indicated in Table III. For Tabun-poisoned muscle this comparison is illustrated in Figures 1 and 2. Muscle was immersed in TMB4 (Fig. 1) and 2-PAM (Fig. 2) prior to incubation by the Koelle technique. Figure 3 shows muscle taken from a control animal sacrificed by open drop ether. Since the amount of CuS precipitate at motor end-plates in Figures 1 and 3 is about the same, one may conclude that TMB4 reactivated ChE to a level similar to that of the control muscle. The lesser reactivation by 2-PAM is shown in Figure 2.

Discussion

The dose of anticholinesterase used to kill the rats was determined solely by a desire to avoid recovery from any inhibitory effect on ChE activity. Whenever possible, therefore, doses were used which would cause death within an hour. Following the initial injection, if marked signs of poisoning did not appear within 10 minutes, a second (and third) injection was given. The total dose varied with the agent used.

Results presented in this paper show that following fatal poisoning in rats by some anticholinesterases, including two insecticides, considerable spontaneous reactivation of ChE occurred post mortem in cadavers kept at room temperature. This temperature range was chosen in an attempt to approximate that which usually prevails before necropsy. At these temperatures the amount of inhibition in muscle samples depended not only upon which anticholinesterase had been used but also upon the time lapse between death and necropsy. Consequently, when one is testing tissues in cases of suspected poisoning by

† Number of injections indicated by figure in parentheses.
‡ Grade in parentheses applies to only a few motor end-plates.

TABLE II

Reversal of Anticholinesterase Inhibition by 2-PAM

Method of sacrifice	Animal no.	Total dose (mg. per kg.)	Time interval: From exposure to death	Time interval: From death to removal of tissue	Kind of muscle	CuS deposit at motor end-plates: Tissue kept frozen until incubation	CuS deposit at motor end-plates: 30 min. immersion in 2-PAM (1×10^{-3} M) before incubation
Diazinon	1	200 (1)*	13 min.	5 min.	Intercostal	+	+++, ++++
	2	200 (1)	13 min.	5 min.	Gastrocnemius	+, ±	+++
Parathion	1	127 (1)	15 min.	2 hr.	Gastrocnemius	+	+++
	2	127 (2)	150 min.	2 hr.	Gastrocnemius	+	+++, ++
Sarin	1	0.225 (2)	30 min.	2 hr.	Intercostal	+	+++
DFP	1	5.0 (2)	13 min.	5 min.	Gastrocnemius	o	+
	2	5.0 (2)	13 min.	24 hr.	Intercostal	o	±, +
	3	5.0 (2)	14 min.	2 hr.	Intercostal	o	+

* Number of injections indicated by figure in parentheses.

TABLE III

Comparative Effectiveness of TMB4, 2-PAM, and Saline in Reversing Inhibition of Cholinesterase

Method of sacrifice	Animal no.	Total dose (mg. per kg.)	Time interval: From exposure to death	Time interval: From death to removal of tissue	Kind of muscle	CuS deposit at motor end-plates after 30 min. pre-incubation immersion in oxime (1×10^{-3} M): TMB4	2-PAM	Saline only
Tabun	II	1.25 (3)*	90 min.	2 hr.	Intercostal	+++, (++++)†	+, ++	o, (+)
	II	1.25 (3)	90 min.	24 hr.	Intercostal	+++, ++	+	±
	III	1.00 (2)	61 min.	2 hr.	Intercostal	+++	+, (++)	+, ±,
Sarin	II	0.225 (2)	50 min.	2 hr.	Triceps	+++, ++	++, +	++‡
DFP	II	5.0 (2)	14 min.	2 hr.	Intercostal	+++, ++	+	o

* Number of injections indicated by figure in parentheses.
† Grade in parentheses applies to only a few motor end-plates.
‡ Recorded as + when immersion in saline was omitted.

anticholinesterases, one should remember that slightly inhibited deposition of CuS might not be an accurate indication of the amount of inhibition that existed at the time of death. The probability of such inaccuracy is negligible in cases of poisoning by the more irreversible anticholinesterases such as DFP.

With the Koelle technique ChE was visualized through hydrolysis of the substrate by the enzyme and subsequent precipitation of CuS at the site of the enzyme. When no CuS was precipitated, the site was not visualized. Provided that the tested sample included motor end-plates, lack of CuS precipitate may be interpreted as an indication of very marked or even total inhibition of ChE activity. In order to be fairly certain that a sample of striated muscle includes motor end-plates, the fibers should be approximately 5 mm. long (6 to 10 mm. for many human muscles). Each fiber has at least one motor end-plate. The chief reason for choosing intercostal muscle is that one is almost certain to include motor end-plates (which lie about midway along the fibers) if the fibers are cut close to each rib. Now that oximes are available, definite proof as to whether sites of ChE actually are present, even though there is no CuS precipitate, can be obtained. Used in suitable concentrations prior to the Koelle test, oximes will reactivate inhibited ChE to the extent of permitting some activity, if not a great deal, and some CuS will be deposited, thus revealing the site of the enzyme. If, under these circumstances, no CuS is deposited, one may conclude that the sample did not include sites of ChE activity.

As a consequence of what is known now, it seems advisable, in cases of suspected poisoning by anticholinesterase, to run two tests of tissue samples, as follows: The teased muscle should be divided into two portions, one of which is immersed in a solution of oxime prior to incubation. Then the site of ChE activity can be seen in the oxime-treated portion and evidence of ChE inhibition, if any, in the other portion. If no CuS is deposited in either portion, the tissue lacks sites of ChE, and no information about activity of the enzyme can be obtained. Although possible, it is considered unlikely that one of the two portions will include motor end-plates and the other none at all, especially if a portion consists of more than a hundred fibers. Spontaneous reactivation will be held to a minimum if the portion of tissue which is not immersed in the oxime is kept cold until the Koelle test is begun. The concentration of oximes used in the experiments was 1×10^{-3} M. This is sufficiently low to avoid anticholinesterase effects by 2-PAM itself.[9,12,13] At this selected concentration, TMB_4 was more effective than 2-PAM.

‡ Recorded as + when immersion in saline was omitted.

Summary

Following death from the anticholinesterases Diazinon, Parathion, tetraethylpyrophosphate (TEPP), Sarin, Tabun, and diisopropyl fluorophosphate (DFP), deposition of copper sulfide (CuS) was partially or markedly inhibited at motor end-plates in striated muscle removed within two hours after death.

In muscle removed from these same animals 24 hours after death, there was considerable deposition of CuS except following death from Tabun or DFP.

The use of an oxime as an adjunct to the Koelle test increased the amount of CuS deposited in post-mortem muscle which showed marked or total inhibition when it was not used.

In the concentration used, TMB_4 was more effective than 2-PAM in reversing inhibited ChE in post-mortem striated muscle of the rat.

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We wish to thank Robert D. Austin for the photographs.

[*Illustrations follow*]

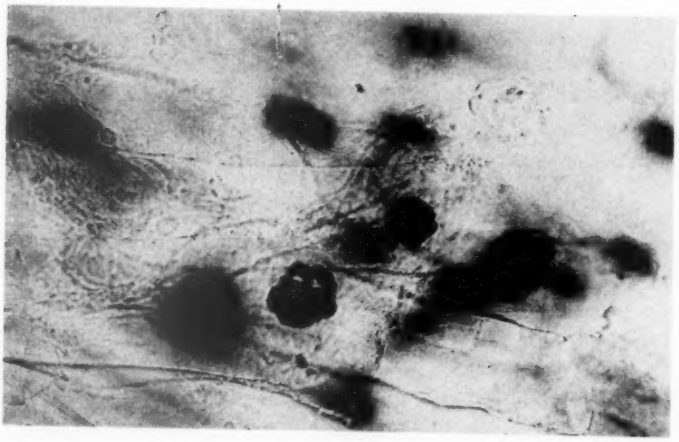
LEGENDS FOR FIGURES

The illustrations represent rat intercostal muscle, with the subneural apparatus of motor end-plates, the site of cholinesterase activity, visualized by CuS precipitate according to the Koelle histochemical technique. The amount of ChE activity was assessed by the amount of deposit; uninhibited activity was denoted as ++++. Muscle incubated for 15 minutes at 37° C. × 160.

FIG. 1. Rat sacrificed by Tabun (1.25 mg. per kg.). Death occurred 90 minutes after first injection. Muscle removed 2 hours after death. Immersed in saline solution of TMB4 (1×10^{-8} M) for 30 minutes before histochemical tests. ChE activity recorded as +++ and ++++. Two motor end-plates in focus show detail of subneural apparatus.

FIG. 2. Same muscle as above but immersed in saline solution of 2-PAM (1×10^{-8} M). Reduced activity recorded as + and ++.

FIG. 3. Rat sacrificed by open drop ether. Immediate necropsy. No pre-incubation immersion. ChE activity recorded as +++ and ++++.



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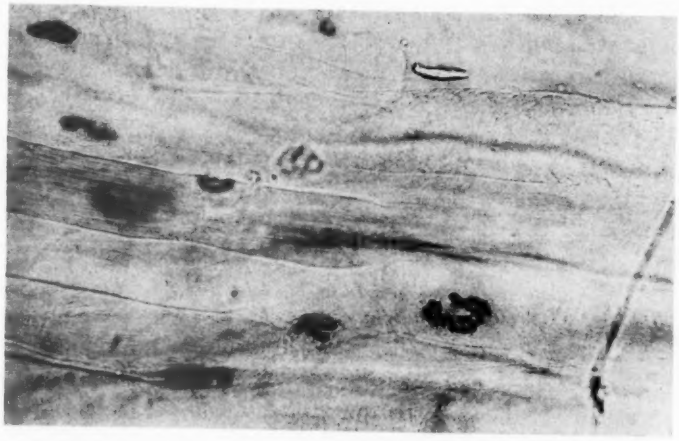

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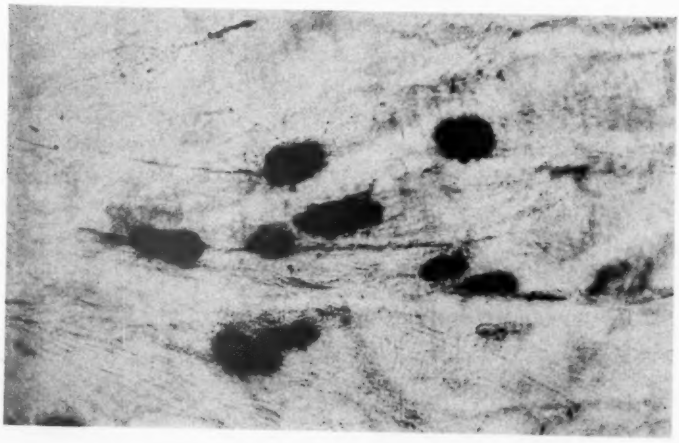

3

THE REMOVAL OF CARTILAGE MATRIX *IN VIVO* BY PAPAIN

Prevention of Recovery with Cortisone, Hydrocortisone and Prednisolone by a Direct Action on Cartilage *

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The effect of cortisone on the synthesis of chondroitin sulfate has been studied by several investigators, mainly by measurement of its influence on the incorporation of sulfur^{35} *in vivo*. When sulfur^{35} is administered to rats in the form of inorganic sulfate, most of what is retained by the animal is incorporated into sulfated mucopolysaccharides, including chondroitin sulfate.[1,2] Layton[3] found that cortisone caused reduction of sulfate fixation in the skin of rats given $Na_2S^{35}O_4$. This was confirmed by Boström and Odeblad, who, in addition, demonstrated an inhibition of sulfur^{35} incorporation into chondroitin sulfate in costal cartilage of cortisone-treated rats.[4] These authors interpreted their results as probably indicating an interference by cortisone upon the exchange of the ester sulfate group of chondroitin sulfate. More recently, however, Schiller and her colleagues have presented evidence indicating that inhibition of sulfate incorporation reflects diminished synthesis of the entire polysaccharide molecule.[5,6]

The depletion of cartilage matrix produced *in vivo* by papain provides a useful system for the investigation of the effects of cortisone and other steroids on the formation of chondroitin sulfate in cartilage. In a previous report,[7] it was shown that the intravenous injection of crude papain into young rabbits was followed within several hours by progressive loss of rigidity and collapse of the ears. This was the result of alterations in cartilage, characterized histologically by the disappearance of the basophilic staining property of matrix and the attainment of a uniformly eosinophilic appearance. The alteration occurred in all cartilaginous tissue throughout the body, including that in the ear, trachea, epiphysis, and articular surface. Spicer and Bryant described loss of metachromasia of cartilage following the injection of crude papain.[8] Bryant, Leder and Stetten demonstrated chondroitin sulfate in the blood and urine of rabbits receiving injections of crude papain.[9] Tsaltas showed that there was considerable reduction in the chondroitin sulfate content of cartilage in rabbits after injection of papain.[10] It was found that the changes in cartilage could be induced by crystal-

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line papain protease, and this enzyme was considered to be the component in crude papain responsible for the phenomenon.[11]

Restoration of cartilage matrix proceeded quickly in normal rabbits. Within 3 to 5 days after injection, the ears recovered their normal tone and erect state.[7] Basophilic substance began to reappear in cartilage matrix within a few days and gradually returned to normal amounts. The time required for recovery of the normal histologic appearance of cartilage matrix varied in different locations in the body, but was completed within 22 days. On the other hand, it was shown previously that cortisone given daily, following an injection of papain, prevented recovery of the normal tone and erect state of the ears for as long as 4 weeks.[7]

The present study was undertaken to investigate the histologic effects of prolonged systemic administration of cortisone and hydrocortisone upon the restoration of cartilage matrix following an injection of papain, and the effects of such treatment on endochondral bone formation. In addition, the influence of the direct action of cortisone, hydrocortisone and prednisolone on the restoration of matrix in articular cartilage was studied.

Material and Methods

Papain was obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio, as the crude powder prepared from dried latex. Solutions were prepared by grinding 0.1 gm. of powder in 10 ml. of saline with mortar and pestle, followed by paper filtration. Kjeldahl determination and measurements by the biuret method showed such solutions to contain 4 to 5 mg. of protein per ml. Cortisone acetate (Merck and Company), hydrocortisone acetate (Merck) and prednisolone acetate (Schering Corporation) were obtained as aqueous suspensions. For intra-articular injections, dilutions in physiologic saline to a final concentration of 1 mg. per ml. were prepared immediately before use.

Young albino rabbits of both sexes, weighing less than 1,000 gm., were used. Large rabbits had been found to be relatively resistant to the effects of papain.[7] Tissues were fixed in 10 per cent neutral formalin, and hematoxylin and eosin stained sections were prepared. Bones were decalcified in dilute formic acid.

Sulfur[35] was administered as $Na_2S^{35}O_4$ in a normal saline solution in amounts described below. Autoradiographs were prepared from paraffin-embedded sections cut at 5 μ, using Kodak contrast process ortho film, according to the method of Dziewiatkowski,[12] and by the stripping film technique of Pelc,[13] using autoradiographic stripping plates AR 10 (Kodak, Ltd.).

Experimental Procedures

Prevention of Restoration of Cartilage Matrix by Cortisone

In order to investigate the consequences of prolonged cortisone administration after an injection of papain, the following experiment was performed. Sixteen rabbits were divided into 4 groups: group 1 received 5 mg. of crude papain; group 2 received 5 mg. of crude papain and, beginning at the same time, daily intramuscular injections of 5 mg. of cortisone for 21 days; group 3 received daily intramuscular injections of 5 mg. of cortisone for 21 days; and group 4 received no treatment. All rabbits were sacrificed on the 22nd day.

All the rabbits given papain exhibited loss of rigidity and collapse of their ears, a condition which reached its greatest extent within 16 hours. The rabbits in group 1, which received papain only, showed recovery of normal tone and erect state of their ears by the fourth or fifth day. In contrast, none of the rabbits receiving cortisone after papain (group 2) regained normal rigidity of their ears during the 22 days of observation. However, 2 rabbits in this group showed a slight return of rigidity after the twelfth day. In the rabbits given cortisone alone (group 3), there were no ear changes except that one rabbit developed slight curling of the tips of the ears on the tenth day. This did not become more marked in the following 12 days. All of the rabbits treated with cortisone (groups 2 and 3) either lost weight or gained slightly compared with the control rabbits (group 4).

Roentgenograms taken on the 15th day of the experiment revealed marked thinning of the epiphysial plates in the rabbits given cortisone after papain (group 2; Fig. 1). No such change was seen in the rabbits given cortisone only (group 3; Fig. 2) or in the rabbits given papain only (group 1).

Histologic examination showed that in the rabbits given papain only (group 1), complete restoration of the normal appearance of cartilage had occurred in all locations examined, including the ear, trachea, joint surface and epiphysis. The intensity of the basophilic staining of the cartilage matrix did not differ from that in the control group (Fig. 3).

In contrast, the rabbits treated with cortisone following papain (group 2) showed either complete absence of basophilia of cartilage matrix (Fig. 4) or only slight basophilic staining of matrix and cartilage cells, the matrix generally having an eosinophilic appearance. The alterations in cartilage were about the same as those ordinarily seen 24 to 48 hours after an injection of papain. In addition, there were marked abnormalities at the sites of endochondral bone growth. Sections taken from the lower end of the femur and upper end of the tibia

showed extreme narrowing of the epiphysial plates. There were a lack of orderly arrangement of cartilage cells into columns and an absence of a zone of maturing cartilage cells. There was virtually complete absence of developing metaphysial trabeculae (Fig. 6). A few trabeculae were present in the peripheral portions of the shaft; these were probably formed prior to cortisone administration.

The rabbits given cortisone only (group 3) showed equivocal diminution in the basophilic staining of cartilage matrix. There was, however, definite retardation of endochondral bone formation. The epiphysial plates showed slight narrowing, and there was moderate reduction in the number of newly formed metaphysial trabeculae as compared with controls (Figs. 5 and 7). This change was not nearly so severe as in those rabbits given a prior injection of papain.

Recovery from the Effects of Cortisone Treatment after Papain

In order to learn whether papain-treated rabbits would recover from the profound disturbance of endochondral bone growth produced by prolonged cortisone administration, the following experiment was undertaken: 12 rabbits averaging 1,000 gm. in weight were divided into 3 groups. Group 1 received 5 mg. of crude papain followed by daily intramuscular injections of 10 mg. of cortisone for 15 days; group 2 received 10 mg. of cortisone daily for 15 days; and group 3 received no treatment. After the 15th day, no treatment was given to any of the groups. All of the rabbits were sacrificed on the 65th day.

The rabbits receiving injections of papain (group 1) developed ear collapse which persisted for 5 to 7 days following the cessation of cortisone treatment. There were no ear changes in groups 2 or 3.

On the 15th day, roentgenograms were taken of all groups. Only the rabbits in group 1 (papain followed by cortisone) showed abnormalities. Marked narrowing of the epiphysial plates was seen in all 4 rabbits. At necropsy on the 65th day, these rabbits (group 1) showed complete recovery of cartilage and had normal-appearing epiphyses and endochondral bone formation. The latter appeared slightly more active than in the control group. The rabbits given cortisone alone (group 2) showed no abnormalities.

Prevention of Restoration of Cartilage Matrix by Hydrocortisone Administered after Papain

To determine whether hydrocortisone would also prevent recovery of papain-affected cartilage, the following experiment was performed. Sixteen rabbits were given injections of 5 mg. of crude papain, and at the same time daily intramuscular injections of 5 mg. of hydrocortisone

were started in half of the rabbits and continued for 16 days. All of the rabbits developed complete collapse of their ears. The rabbits given papain alone showed recovery of their ears by the fifth day. Three of the rabbits given hydrocortisone died during the experiment, on the fourth, tenth and 14th days, without any recovery of ear rigidity prior to death. None of the remaining 5 rabbits showed gross ear recovery during the 16 days of injections. All rabbits were sacrificed on the 16th day.

The cartilage from the rabbits receiving papain and hydrocortisone was eosinophilic except for a small amount of basophilic substance in and around chondrocytes. There was marked narrowing of epiphysial plates and reduction of endochondral bone formation. In rabbits given papain only, cartilage in the ear and epiphysial plates was normal histologically. Sections of tracheal and articular cartilage, however, showed that recovery was not yet complete in these locations. There was proliferation of cartilage cells, often in clusters, surrounded by intensely basophilic material. The small amount of intervening matrix was almost completely eosinophilic (Fig. 8).

Local Prevention of Restoration of Cartilage Matrix by Intra-articular Injections of Cortisone, Hydrocortisone or Prednisolone

In order to determine whether the inhibitory effect of corticosteroids could be produced locally, in the absence of systemic factors, intra-articular injections of steroids were given after papain had been administered. In preliminary experiments, it was learned that injections of cortisone, hydrocortisone or prednisolone in doses of 0.1 mg. daily into one knee joint did not significantly delay recovery of ear cartilage. The following experiment was then performed. Each of 16 rabbits received a single intramuscular injection of 5 mg. of crude papain. Twenty-four hours later, daily injections were given into the right knee joint as follows: 4 rabbits received 0.1 mg. cortisone; 4 rabbits received 0.1 mg. hydrocortisone; and 4 rabbits received 0.1 mg. prednisolone. These 12 rabbits were given 0.1 ml. of saline into the left knee joint daily. The remaining 4 rabbits received no treatment following the injection of papain.

The rabbits given intra-articular cortisone or hydrocortisone showed a slight delay in return to the normal gross appearance of their ears. The recovery time averaged 6 to 7 days, which was 2 days longer than in the rabbits receiving only papain. The rabbits receiving intra-articular injections of prednisolone recovered in the usual period of 4 to 5 days.

The rabbits were sacrificed on the twelfth day following the administration of papain. In all of the rabbits given intra-articular corticosteroids, there were gross differences between the articular cartilage of the right and left knee joints. The joints injected with steroids showed thinner and more translucent articular cartilage than the opposite joints. This difference was most marked in the group receiving prednisolone (Fig. 9). The rabbits given papain only had grossly normal joints.

Hematoxylin and eosin stained sections showed that in the saline-injected joints there was marked restoration of matrix of the surface cartilage in every case. There was proliferation of cartilage cells, often in the form of columns, which were perpendicular to the surface. The chondrocytes were intensely basophilic (Figs. 10 and 12). The basophilic staining of the matrix was almost as intense as in control rabbits.

In contrast, the surface cartilage of the steroid injected joints showed little or no restoration of matrix. There was no proliferation of cartilage cells. In joints injected with cortisone or hydrocortisone, there was slight basophilic staining especially in and around chondrocytes (Fig. 13), but in those injected with prednisolone, there was complete absence of basophilic staining (Fig. 11).

The epiphysial cartilage of the lower ends of both femurs from all groups was normal. The ear and tracheal cartilage showed proliferation of basophilic cartilage cells, which were surrounded by intensely basophilic substance, although some of the matrix was still eosinophilic.

In the rabbits receiving papain only, articular and epiphysial cartilage was normal histologically. The ear and tracheal cartilage was similar to that in the rabbits given intra-articular injections of steroids.

The Effect of the Intra-articular Administration of Prednisolone on Incorporation of Sulfur^{35} into Articular Cartilage

The histologic observations described above indicated that cortisone, hydrocortisone and prednisolone interfered with chondroitin sulfate synthesis in the cartilage of joints injected with these steroids. In order to obtain further information concerning this, sulfur^{35} was used. It has been shown that when sulfur^{35} is administered to animals in the form of $Na_2S^{35}O_4$, a large percentage of it is rapidly excreted, and most of the sulfur^{35} retained in the animal is incorporated into sulfated mucopolysaccharides.[1,2] In cartilage, virtually all of the sulfur^{35} is in the form of chondroitin sulfate.[1] Prednisolone was chosen because, of the 3 steroids tested, it resulted in the most marked inhibition of local recovery.

Each of two 1,000-gm. rabbits was given an intramuscular injection of 5 mg. of crude papain; this produced typical ear collapse. On the following day each rabbit received an injection of 0.1 mg. of prednisolone into the right knee joint and 0.1 ml. of saline into the left knee. This was repeated daily for 7 days. On the fourth day following papain injection, each rabbit was given an intravenous injection of 4 mc. of sulfur^{35} as $Na_2S^{35}O_4$, and both rabbits were sacrificed 3 days later.

The surface cartilage of the prednisolone-injected joints was slightly thinner and more translucent than that of the opposite joints. Hematoxylin and eosin stained sections showed no basophilic substance in the articular cartilage of the prednisolone-injected joints, in contrast to the opposite, saline-injected joints where there was moderate accumulation of basophilic substance in and around chondrocytes. Much of the matrix was still eosinophilic (Figs. 14 and 15).

Autoradiographs were prepared of the lower end of the femur and upper end of the tibia. The intensity of the image over the articular cartilage was considerably less in the prednisolone-injected joints than in the opposite joints (Figs. 16 to 19). The autoradiographs prepared with stripping film showed that sulfur^{35} was present both intracellularly and in the matrix. The relative amounts of intra- and extracellular substance did not differ in the prednisolone and saline injected joints (Figs. 18 and 19).

Discussion

The results of the present study indicate that cortisone, hydrocortisone and prednisolone interfered with the synthesis of chondroitin sulfate in cartilage. This was evidenced by the fact that these substances prevented the recovery of normal cartilage tone and the reaccumulation of normal amounts of basophilic matrix following its depletion by papain. It has been shown that papain results in a considerable loss of chondroitin sulfate from cartilage,[9,10] and this is reflected by the disappearance of basophilic substance from cartilage matrix and loss of cartilage tone. The present observations are consistent with the interpretation that the inhibitory effect of cortisone on sulfur^{35} fixation in animals[3,4] represents an inhibition of synthesis of chondroitin sulfate rather than mere sulfate exchange. Schiller and Dorfman[6] found that cortisone resulted in decreased incorporation of C^{14} and S^{35} into chondroitin sulfate and that hydrocortisone treatment was followed by diminution in the turnover rate of C^{14} and S^{35} in the chondroitin sulfate of rat skin. Schiller, Mathews, Cifonelli and Dorfman[5] had previously demonstrated that the sulfate group of chondroitin sulfate turned over at the same rate as galactosamine and the

N-acetyl group and concluded therefore that sulfate incorporation reflected synthesis of the entire polysaccharide molecule rather than sulfate exchange.

The observation that local cartilage recovery could be prevented by intra-articular injections of cortisone, hydrocortisone or prednisolone, in amounts too small to prevent recovery of cartilage elsewhere in the body, demonstrated that the interference with synthesis of cartilage matrix was the result of a direct action on cartilage rather than the result of a general metabolic disturbance or other systemic factors. It is not certain that this effect was the result of the steroid molecule as such, or of one of its breakdown products, since it has been shown that hydrocortisone was rapidly converted into a variety of substances following intra-articular injection.[14]

The effects of corticosteroids on the incorporation of sulfur^{35} by cartilage slices *in vitro* are of interest in this connection. Boström and Odeblad[4] reported that the uptake of S^{35} by cartilage slices was diminished after incubation with cortisone. Clark and Umbreit[15] confirmed this, but found that hydrocortisone or its acetate ester increased the S^{35} uptake in cartilage slices. These authors concluded that the direct effects of corticosteroids *in vitro* bore no relationship to their effect in intact animals. They suggested that the diminished synthesis of chondroitin sulfate *in vivo* might be secondary to other changes and not due to direct inhibition of chondroitin sulfate synthesis by cortisone. The results observed in the present study following the intra-articular injection of steroids demonstrated that each of the steroids inhibited the synthesis of chondroitin sulfate by a direct action on cartilage. The possibility exists, however, that the effects were mediated through a breakdown product of the steroids which was not always produced in the *in vitro* system.

When sulfur^{35} was administered to an animal, it first appeared within cartilage cells, and during the course of the next several days it was transferred from the cells to the cartilage matrix.[16] The autoradiographic observations in this study showed that although less sulfate was incorporated into the cartilage of the joint injected with prednisolone than in the control joint, there was no interference with its deposition in the extracellular matrix. Thus, no evidence was provided to indicate that this steroid acted by interfering with transfer of material through the cell membrane, although such a mechanism could not be excluded. The mechanism by which the adrenal steroids interfere with the synthesis of chondroitin sulfate is unknown.

In rabbits given papain without steroids and allowed to recover normally, there was a marked proliferation of chondrocytes in and around which basophilic substance accumulated. This represented newly synthesized chondroitin sulfate. In steroid-treated rabbits, not only did the basophilic material fail to accumulate, but the proliferation of cartilage cells was also prevented, indicating that the effect of these steroids on cartilage was not merely a selective interference with polysaccharide synthesis.

The prolonged administration of cortisone or hydrocortisone to young rabbits did not result in unequivocal histologic alterations in cartilage except in the epiphysis, where slight thinning of the epiphysial plate and retardation of new bone formation was seen. These changes are somewhat similar to those described by Follis in growing rats given cortisone.[17] If an injection of papain was given prior to prolonged cortisone or hydrocortisone administration, the effects on epiphysial bone formation were markedly intensified. The epiphysial plates became extremely thin and the usual zones of cartilage cells were no longer apparent. It is remarkable that if the steroid injections were stopped, such a deformed epiphysial plate regained its normal structure and function.

Summary

The restoration of the normal rigidity of cartilage and the reaccumulation of basophilic substance in cartilage matrix following its *in vivo* depletion by papain were largely prevented by cortisone, hydrocortisone or prednisolone. Recovery of cartilage could be prevented locally in joint surfaces by the intra-articular injection of cortisone, hydrocortisone or prednisolone. Of the 3 steroids tested, prednisolone was the most effective in preventing local restoration of cartilage matrix. The intra-articular administration of prednisolone resulted in diminished uptake of systemically administered sulfur[35] by the cartilage of the injected joint.

These results indicate that cortisone, hydrocortisone and prednisolone inhibit the synthesis of chondroitin sulfate in cartilage as the result of a direct action on cartilage.

The prolonged systemic administration of cortisone or hydrocortisone to young rabbits resulted in slight thinning of epiphysial plates and reduction in new bone formation. Prolonged treatment of young rabbits with cortisone or hydrocortisone following an injection of papain resulted in marked thinning and deformity of epiphysial plates and cessation of endochondral bone formation. Following cessation of steroid therapy, normal endochondral bone formation was resumed.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Roentgenogram of lower femur and upper tibia of rabbit after 15 daily injections of 5 mg. of cortisone following an injection of crude papain. The epiphysial plates are closed. Compare with Figure 2. × 1½.

FIG. 2. Roentgenogram of lower femur and upper tibia of rabbit after 15 daily injections of 5 mg. of cortisone. The epiphysial plates are of normal appearance (best seen in tibia). Compare with Figure 1. × 1½.

FIG. 3. Section of tracheal cartilage from rabbit sacrificed 21 days following an injection of papain. The matrix shows a normal amount of basophilic substance. Hematoxylin and eosin stain. × 100.

FIG. 4. Section of tracheal cartilage from rabbit given 21 daily injections of 5 mg. of cortisone following an injection of papain. The cartilage is devoid of basophilic substance. Hematoxylin and eosin stain. × 100.

FIG. 5. Section of epiphysis of lower end of femur from control rabbit. Note the thickness of the epiphysial plate and the numerous proliferating bony trabeculae. Hematoxylin and eosin stain. × 70.

FIG. 6. Section of epiphysis of lower end of femur from rabbit given 21 daily injections of 5 mg. of cortisone following an injection of papain. The epiphysial plate is thin, and the orderly arrangement of cartilage cells is lacking. There are no newly formed trabeculae. Hematoxylin and eosin stain. × 70.

FIG. 7. Section of epiphysis of lower end of femur from rabbit given 21 daily injections of 5 mg. of cortisone. The epiphysial plate is narrowed but shows the usual zones of cartilage cells. There is reduction in the number of newly formed trabeculae. Hematoxylin and eosin stain. × 70.

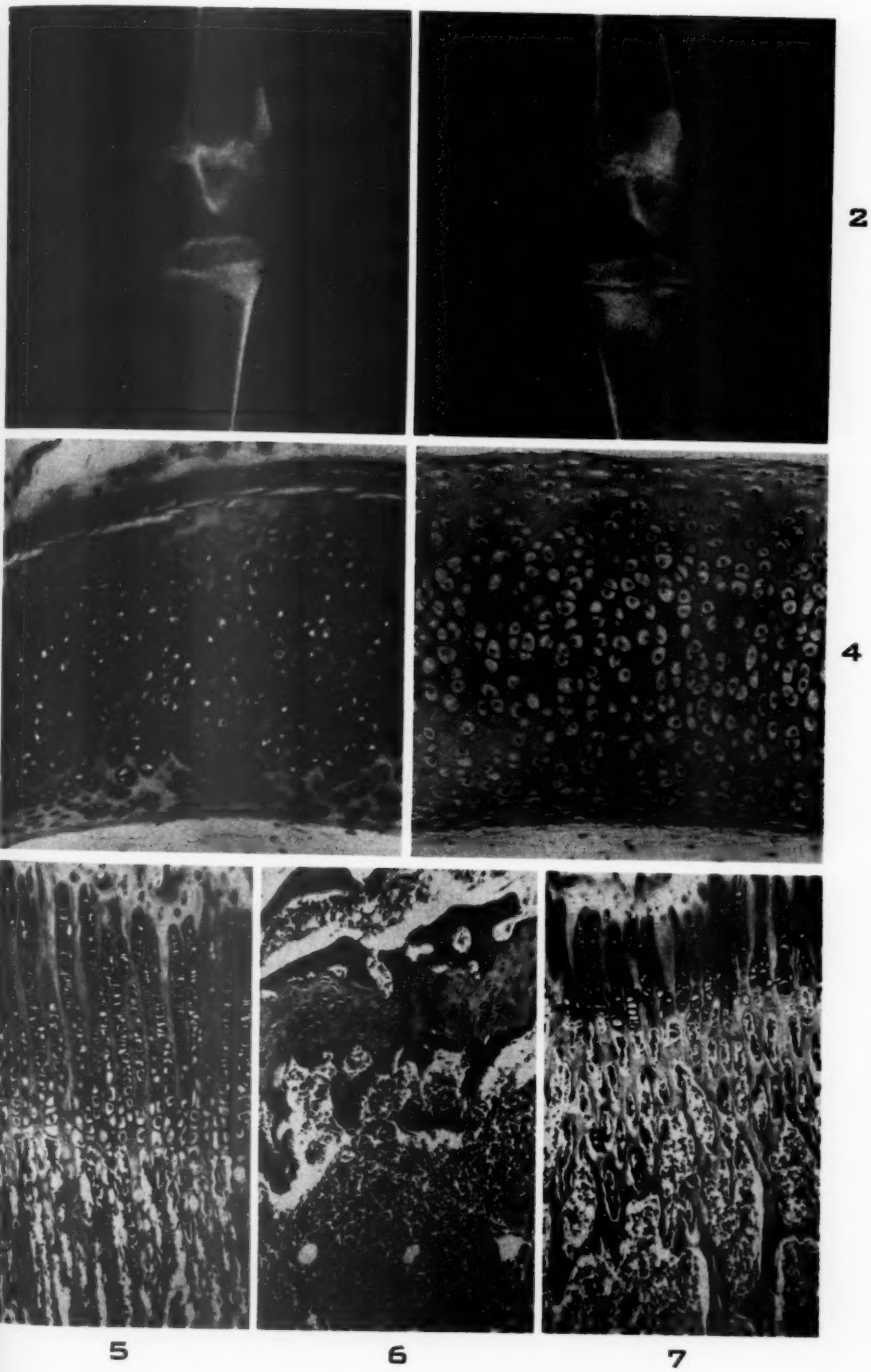
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FIG. 8. Tracheal cartilage from rabbit sacrificed 16 days after an injection of papain. There is proliferation of cartilage cells to form clusters. The chondrocytes and the immediately surrounding matrix are intensely basophilic. There is a small amount of intervening matrix which is still eosinophilic. Hematoxylin and eosin stain. × 60.

FIG. 9. Lower ends of the left and right femur from a rabbit given 12 daily injections of 0.1 mg. of prednisolone into the right knee joint following an injection of papain. The prednisolone-injected joint has thinner and more translucent cartilage. About natural size.

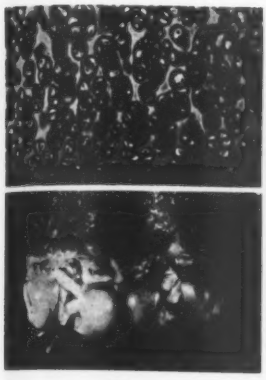
FIGS. 10 and 11. Sections of surface cartilage of left and right femurs from rabbit given papain followed by 11 daily injections of 0.1 mg. of prednisolone into a right knee joint. The saline-injected joint (Fig. 10) shows proliferation of cartilage cells and accumulation of basophilic substance in the matrix. The steroid injected joint (Fig. 11) shows no new cartilage cells or accumulation of basophilic matrix. Hematoxylin and eosin stain. × 60.

FIGS. 12 and 13. Sections of surface cartilage of left and right femurs from rabbit given papain followed by 11 daily injections of 0.1 mg. of hydrocortisone into right knee. The left joint (Fig. 12) shows marked restoration of cartilage matrix compared with the injected joint (Fig. 13) in which only a small amount of basophilic substance is present, especially within chondrocytes. Hematoxylin and eosin stain. × 75.

FIGS. 14 and 15. Sections of surface cartilage of left and right femur from rabbit given papain followed by 7 daily injections of 0.1 mg. of prednisolone into right joint. In the control joint (Fig. 14) there is proliferation of intensely basophilic chondrocytes, but most of the matrix is still eosinophilic. The injected joint (Fig. 15) shows no recovery of cartilage. Hematoxylin and eosin stain. × 75.

FIGS. 16 and 17. Autoradiographs prepared with Kodak contrast process ortho film of lower end of left (Fig. 16) and right femur (Fig. 17) of same rabbit as shown in Figures 14 and 15. On the fourth day the rabbit received sulfur^{35}. Surface cartilage shows a weaker image in the articular cartilage of the steroid injected joint. × 2.

FIGS. 18 and 19. Autoradiographs prepared with stripping film of the articular cartilage of left (Fig. 18) and right (Fig. 19) femur from rabbit given papain followed by 7 daily injections of 0.1 mg. of prednisolone into the right knee joint. On the fourth day the rabbit was given sulfur^{35}. The cartilage in the injected joint shows fewer granules in the emulsion than the control joint. Hematoxylin and eosin stain. × 800.



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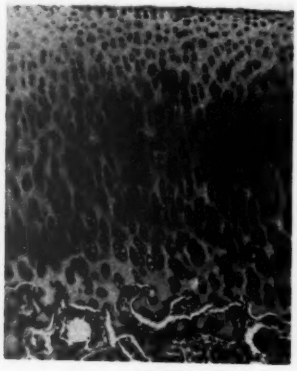

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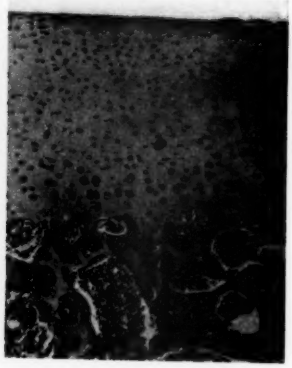

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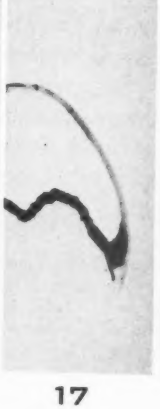

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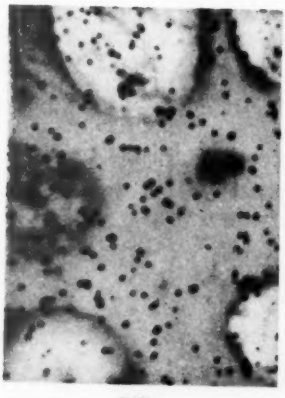

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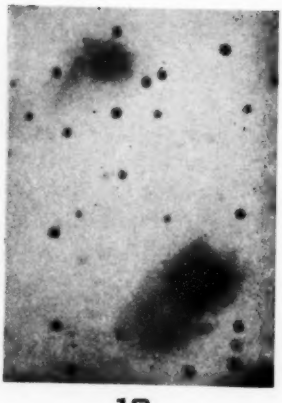

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CELL DEATH

III. The Effect of Injury on Water and Electrolytes of Ehrlich Tumor Cells *

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The role of ions in the cell has not been fully understood. They have been thought to participate as hydration regulators, as integral parts of the structure of the cell,[1] associated with the transport of adenosine triphosphate,[2,3] and as inhibitors or accelerators in specific enzymatic reactions.[4-9] Most studies on cell electrolytes suggest that cells are iso-osmotic although much evidence has been presented to indicate an intracellular hypertonicity.[10] It is also accepted by many that sodium and potassium transport systems are linked and reciprocal in nature;[11] however, many reports are available on the independence of ion and water movements.[12-14] Many of the exchange studies available are concerned with only one of 3 major components: sodium,[15] potassium,[16-18] or water,[19-21] although others include two or more components.[22]

The system of Ehrlich tumor cells used in these experiments offers the opportunity of measuring not only sodium and potassium but, indirectly, the water content as well. It also eliminates two errors of *in vitro* studies using tissue slices, namely, miscalculation of extracellular space and the presence of large amounts of dead or dying cells at the edge of the slice.

Experiments

The cells were obtained by methods described in preceding papers.[23,24] In addition to those already described, the following measurements were made.

Cell Volume

The cells are generally considered to be spheres in the free floating state. In order to maintain this living form uninjured, a few drops of the cell suspension were pipetted on a blood counting chamber. Multiple photographs were taken with a phase microscope at a magnification of 970 times. Since only 6 to 8 cells were visible in each photograph, it was necessary to select 8 to 10 random fields throughout the slide in order to have at least 50 cells per sample. Later, circumferences of the

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cells in the enlarged photographs were plotted by means of a planimeter, and from these, cell volumes were calculated and taken as an indication of water content.

Nuclear Volume

Since there is a very high nuclear cytoplasmic ratio in tumor cells, it is difficult to distinguish the nucleus from the cytoplasm accurately in unstained, uninjured cells. The nuclear size was obtained by planimeter measurements of camera lucida drawings of fixed cells stained with Feulgen or hematoxylin and eosin stains.

Electrolytes

At each time period, five 2-cc. samples were withdrawn from each of 3 Erlenmeyer flasks for sodium and potassium determinations. The cell suspensions, in graduated centrifuge tubes, were spun at 3,000 r.p.m. for 5 minutes in a Clay-Adams centrifuge and the supernatant decanted. The sediment was allowed to drain for 8 hours. At this time 0.5 cc. of concentrated nitric acid was added for digestion, and the tops of the tubes were covered with parafilm. Forty-eight hours later, 1.5 cc. of distilled water was added to restore the samples to their original volume, and the sodium and potassium content was determined in a Model B Beckman Flame Spectrophotometer. Five readings were taken on each specimen.

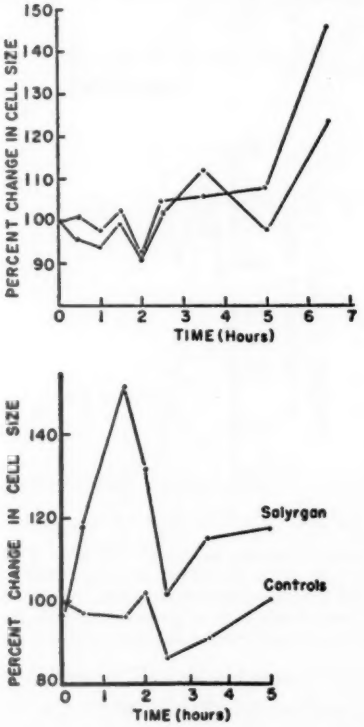
Results

There are several methods of determining cellular volume, none of which are accurate. Perhaps the best is that of Lucké and Parpart,[25] who recorded the changes in light transmitted through a cell suspension directly on a galvanometer. Changes in volume of perfect osmometers will be measured by changes in light transmitted. However, this method demands that the cells always swell homogeneously and that they do not contribute their content to the extracellular suspension. Neither of these stipulations can be met in experiments of long duration. Cell size in these experiments was originally computed by two methods: the microhematocrit method employed by Ponder[26] and others, and a photographic planimetry method described earlier. It was surprising how closely the two methods checked each other in one experiment. However, a word of caution should be voiced concerning the packed centrifuged cells in the microhematocrit chamber. There is little doubt that centrifugation results in damage to the cell membrane which may not show up immediately. In one experiment, cells were initially examined and volume calculated by the planimetry method. A second aliquot was centrifuged in a microhematocrit tube. Follow-

ing centrifugation, the packed cells were resuspended in Krebs-Ringer solution, photographed, and the size of 50 cells measured by means of a planimeter. A third aliquot from the original uncentrifuged suspension was taken for the final control sample and the cell size measured with the planimeter. The membranes of the centrifuged cells were sufficiently damaged so that, on resuspension after centrifugation, they took in 45 per cent more water than the two control samples, before and after centrifugation.

Changes in Cell Size in Injured Cells

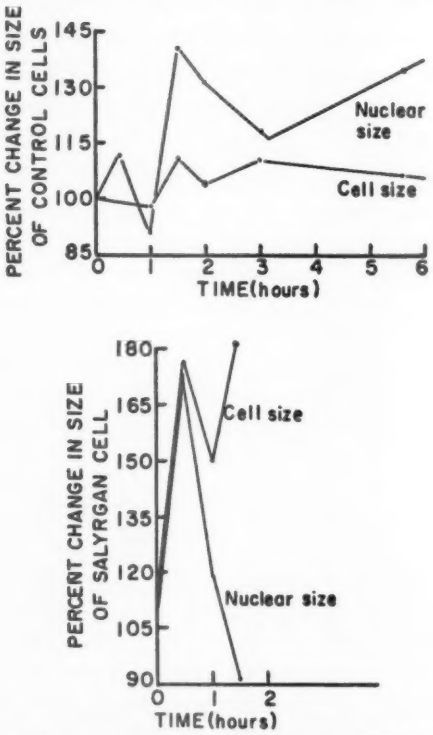
The cells changed their size in a fairly characteristic fashion, regardless of the form of injury. These changes in size were assumed to represent changes in the water volume. The characteristic curves of initial shrinkage followed by a terminal swelling of the cell were found



Text-figure 1. Effect of irradiation and salyrgan on the size of Ehrlich tumor cells. Cells suspended in Krebs-Ringer solution (pH 7.46) with glucose added to a final concentration (0.0014 M). The top graph represents the change in size in the irradiated group, and the bottom graph, in the salyrgan-treated group. The bottom line in each group represents the control sample. Each point represents the average of 50 cell planimeter measurements and the percentage of change from the original cell size plotted.

in all 3 samples: control, irradiated and salyrgan-treated (Text-fig. 1). The only difference was the time element. The salyrgan-treated cells, as expected, started to swell almost immediately, while the irradiated cells had delayed swelling and paralleled the control cells until the terminal phase.

The nuclear size may have no relation to the size of the cell. It varied over a wide range when measured by a planimeter (Text-fig. 2).

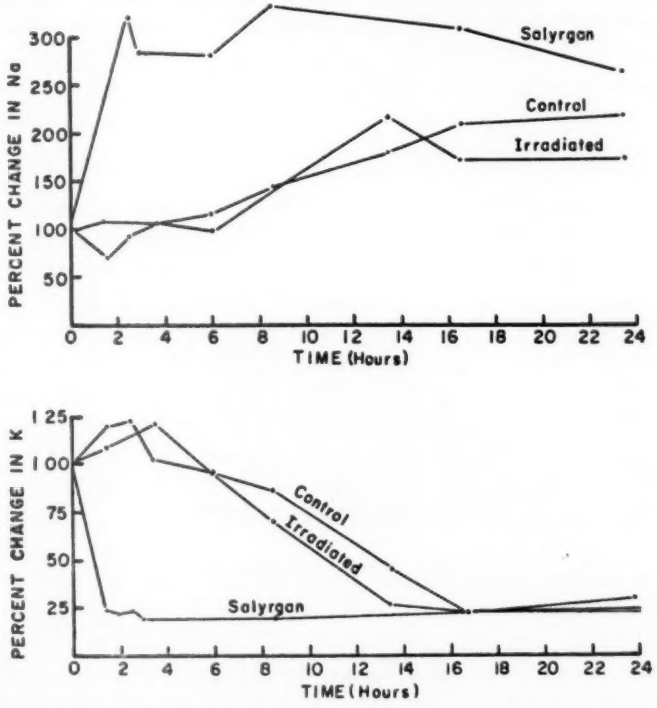


Text-figure 2. Relation of nuclear to cytoplasmic size of Ehrlich tumor cells. Each point represents the average of 50 planimeter measurements. The depression in nuclear size between 1 and 3 hours in the control cells is significant to the 0.05 level. There is no significant change in cell size in the control group by analysis of variance. The changes in nuclear and cell size in the salyrgan-treated cells are significant to the 1 hundredth level.

The nucleus appeared more labile and became significantly smaller or larger without corresponding alterations in the cytoplasm when cells were treated with salyrgan. The nucleus would swell proportionately with the rest of the cell, but the shrinkage of the nucleus might occur independently of changes in the cytoplasm. The latter phenomenon took place dramatically and irreversibly.

Changes in Sodium and Potassium Content in Injured Cells

The sodium and potassium in general maintained a reciprocal relationship to each other; in addition, the sodium closely paralleled the changes in water content. The control tumor cells maintained their normal concentrations for a period of 3 to 6 hours. At this time, concurrently with a decrease in respiration, fermentation, and the production of energy, there were a gradual loss of potassium and an associated increase in the amount of sodium and water contained in the cells (Text-fig. 3). The irradiated cells followed this pattern with slightly



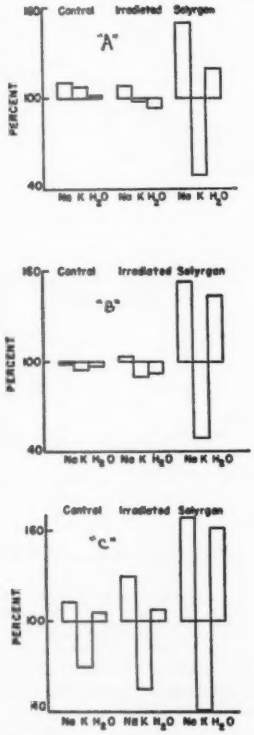
Text-figure 3. The effect of irradiation and salyrgan on the sodium and potassium content of Ehrlich tumor cells. Each point represents the average of 5 samples (5 readings on each sample) in a typical experiment.

earlier terminal alterations. The salyrgan-treated cells immediately reversed the normal intracellular ion content. When the cell had taken up 1.5 to 2.0 times its original content of sodium, it burst, and there was a loss of sodium and water. In experiments of longer duration, the loss of sodium following disruption of the membrane was followed by a secondary uptake of sodium. This was presumably due to the

attachment of hydrated ions to the newly released reactive groups of the denatured protein molecules. The sodium attached to the residual cytoplasmic structure remained for several hours until the protein was gradually dissolved into the surrounding medium. The irradiated and control cells ultimately followed a pattern very similar to that seen in the salyrgan-treated cells.

Correlation of Sodium, Potassium, and Water in Injured Cells

Text-figure 4 provides a summary of 8 experiments, representing hundreds of determinations in the study of electrolyte and water changes in injured cells. The pattern followed was the same, regardless



Text-figure 4. Effect of irradiation and salyrgan on Ehrlich tumor cells. The results are plotted as percentage of change in sodium, potassium, and water content, with the original cell samples given the value of 100 per cent. Part A represents changes during the first hour of incubation. Each bar represents 185 determinations from 7 different experiments. Part B represents the changes during the 1 to 6 hours of incubation. Each bar represents the average of 150 determinations from 6 different experiments. Part C represents changes from the terminal phase of cell death. Each bar represents the average of 150 determinations from 6 different experiments.

of the form of injury. The graph is divided into 3 portions. The top section (A) represents the changes occurring in the initial transfer of cells into cold Krebs-Ringer solution. The salyrgan-treated cells immediately gained sodium and water and lost potassium, whereas the control and irradiated cells underwent an equilibratory period with minor fluctuations of usually less than 10 per cent. The middle section (B) of the graph represents the major portion of the experiment, lasting 5 to 6 hours. While the control cells were maintaining a very constant sodium, potassium, and water content, the irradiated cells were beginning to lose potassium and to undergo the slight shrinkage previously described. In the bottom section of the graph (C) the final picture is plotted. After 5 to 6 hours of incubation, all 3 samples gained sodium and water and lost potassium in amounts reflecting the state of injury to the cell.

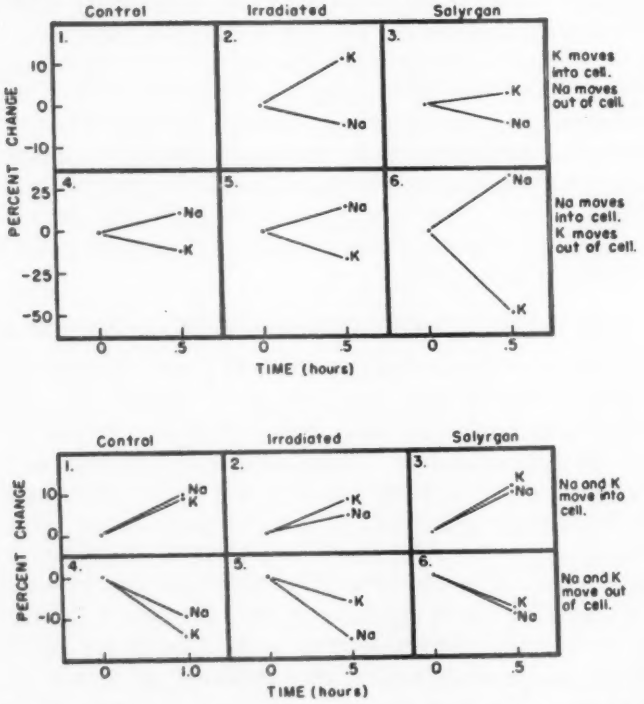
Independence of Sodium, Potassium, and Water Mechanisms

We believe the figures cited in Text-figure 4 show the general picture of sodium, potassium, and water changes in injured cells. However, these very cells made every effort during the process of death to compensate for the irregularities. During this period of stress, the complete independence of the sodium, potassium and water regulating mechanisms was clearly illustrated. Subsequent figures were taken from different experiments at different time periods in successive 30 or 60 minute periods. All results were statistically evaluated by analysis of variance and found to be significant to the 1 per cent or the 5 per cent levels, as indicated. The alterations took place in the control and irradiated groups while trypan blue indicated 95 to 100 per cent viability.

Text-figures 5 and 6 are concerned with the movements of sodium and potassium. As was noted in Text-figure 4, the overall trend was one of a reciprocal nature between these two ions, and, indeed, in the top portion of Text-figure 5 this is easily illustrated. However, in Text-figures 5 and 6 it is also clearly shown that sodium and potassium might move together, or one might not change while marked changes were noted in the other ion.

Text-figures 7 and 8 are concerned with the movements of all 3 major components, sodium, potassium, and water. Again, as previously shown in Text-figure 4, sodium and water usually moved together in the opposite direction from potassium. However, under periods of stress, the 3 components might move completely independently of one another.

The final Text-figure 9 merely indicates that the sodium and potassium changes shown to be associated with cell death occurred earlier and to a more severe degree in those cells maintained under strict anaerobic conditions.

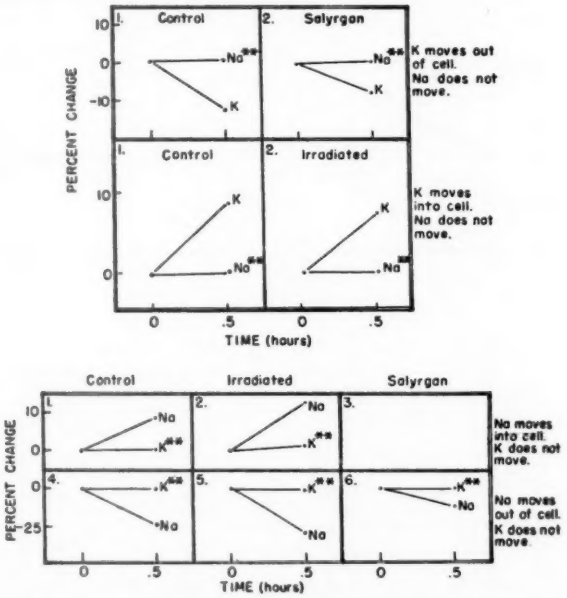


Text-figure 5. Effect of irradiation and salyrgan upon Ehrlich tumor cells. The graphs show that sodium and potassium may either move in a reciprocal fashion or move together concurrently in or out of the cell. The 2 points taken for each ion represent 5 samples taken from the same flask at 2 consecutive time periods 30 minutes apart. The flasks contained control, irradiated, or salyrgan-treated cells from 7 similar experiments, and the samples were taken after varying periods of incubation. The results are plotted as percentage of change in ion content from the original. All results are significant at the 1 hundredth level by analysis of variance except when starred; these are significant at the 5 hundredth level. All cells in the control and irradiated samples show 95 to 100 per cent viability as measured by the entrance of trypan blue into the nucleus.

Discussion

The subject of the passage of ions and water across a cell wall has been extensively reviewed recently.[11,27,28] Although the terms diffusion, active transport, pinocytosis, and phagocytosis are used to describe transport mechanisms, the definitions of these terms are vague, and considerable confusion and overlap result.

Many believe that while sodium is an active transport process,[29] potassium is passive,[30,31] whereas others believe potassium represents active transport also.[20,32-34] Our experiments did not elucidate any new information on this subject but merely confirmed a gain in sodium and loss in potassium under anaerobic conditions.

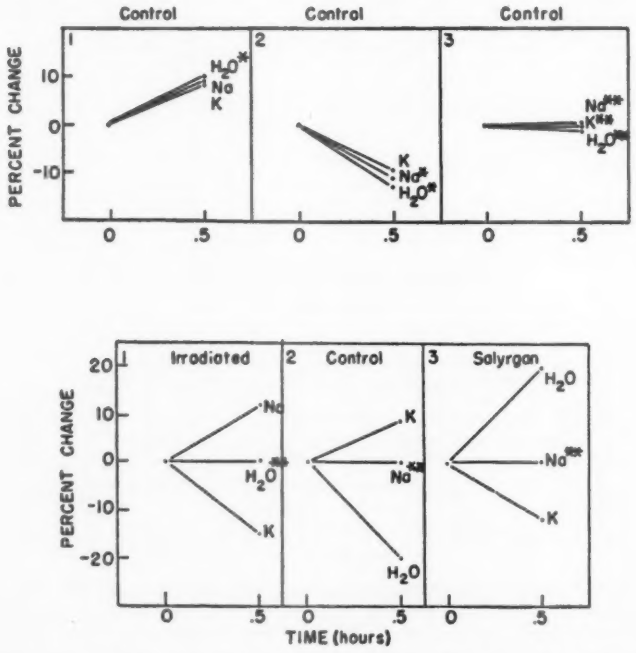


Text-figure 6. Effect of irradiation and salyrgan upon Ehrlich tumor cells. The graphs show that one ion may remain constant while the other ion moves in or out of the cell. The taking of samples, statistical evaluation, and viability tests were done as described in Text-figure 5.

It has been claimed that in all carefully controlled studies of the cell, there is a reciprocal relationship between sodium and potassium.[11] Others have failed to find this relationship constantly.[12,13] It has also been claimed that the sum of the sodium and potassium remains constant regardless of the treatment imposed.[35] Our experiments confirm, in general, the reciprocal nature of sodium and potassium over a long period of time. However, we believe that there is unequivocal evidence, supported by more than adequate statistical evaluation, that in periods of stress with disturbance of the regulatory mechanisms of the cell, sodium, potassium, and water may move in and out of the cell independently of each other. They can even move against concentration gradients and in a manner suggesting that many other factors not usually considered are important in regulation of ion concentrations.

These may include regions of varying metabolic activity,[33,36-39] hormones,[40-42] special tissue characteristics,[43,44] bound ions, inorganic metabolites,[45] or organic metabolites such as amino acids.[16]

We suggest that there are continued changes in the osmotically inactive forms of base in the cell. The water content of particulate components of the cell, such as mitochondria and nucleus, as well as the



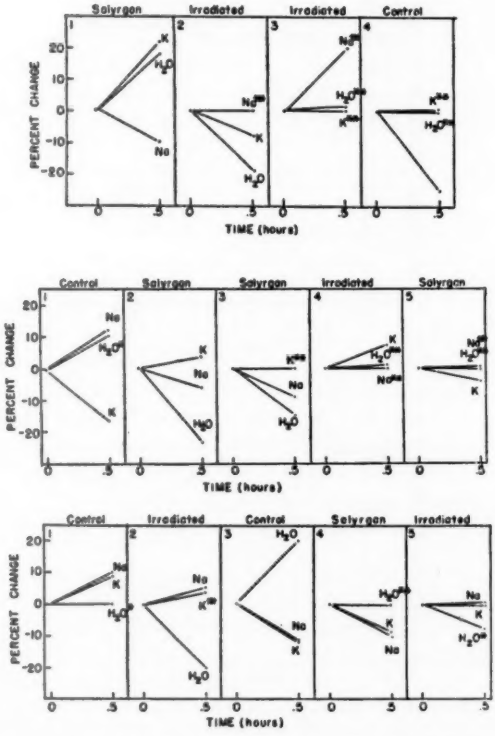
Text-figure 7. Effect of irradiation and salyrgan upon Ehrlich tumor cells. The graphs show that all 3 components measured (sodium, potassium, and water) may move concurrently and also independently. The taking of samples, statistical evaluation, and viability tests were done as described in Text-figure 5.

intramolecular water in corpuscular proteins, may respond primarily to local changes in cell metabolism and secondarily to the regulating influences of the complete cell. The whole series of experiments, including the specific effects on the structure (the variance in nuclear cytoplasmic ratio) as well as function (inhibition of division only), further confirmed this impression.

Effect of Injury on Electrolytes and Water

There are many publications concerning the effect of irradiation on sodium and potassium *in vivo* in the mammalian body,[28,29] and *in vitro*

on yeast and mammalian red blood cells[46] and sarcoma cells.[47] In general, they all show a loss of potassium and increase in sodium. One investigator irradiating amphibian erythrocytes also noted the preliminary shrinkage of the cell before swelling,[48] as we did. Our experiments are interesting in this regard only in that they showed that the control and salyrgan-treated cells exhibited shrinkage before the final swelling similar to that of the irradiated cells at different time intervals.

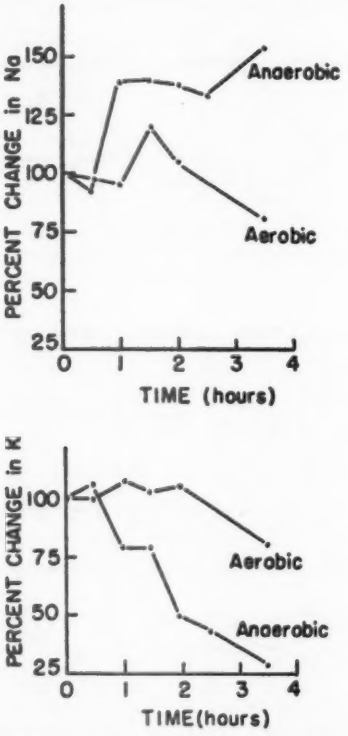


Text-figure 8. Effect of irradiation and salyrgan upon Ehrlich tumor cells. The independence of sodium, potassium, and water is well illustrated in this graph. The taking of samples, statistical evaluation, and viability tests were done as described in Text-figure 5.

Summary

Ehrlich tumor cells, when injured, immediately show small changes (usually not over 10 per cent) in sodium, potassium, and water content. These changes are reversible and are not fatal or detrimental to the fermentative or oxidative metabolism of the cell, although it is possible that they may have a deleterious effect on mitosis. A constant rela-

tionship between sodium, potassium, and water is maintained, but eventually, regardless of the form of injury, the cell swells, loses potassium, and incorporates large amounts of sodium and water. During periods of stress, the mechanism governing sodium, potassium, and water may be altered so that these 3 components may move independently of each other.



Text-figure 9. Effect of irradiation and saylrgan upon Ehrlich tumor cells. Effect of anaerobic conditions on the sodium and potassium content of Ehrlich tumor cells. Each point represents an average of 25 determinations from a typical experiment.

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AGING AND OSTEOARTHRITIS OF THE HUMAN STERNOCLAVICULAR JOINT*

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The aim of the present investigation was to analyze the role of articular aging in the pathogenesis of osteoarthritis, a problem which has been under discussion for a long time.[1-6] Moreover, it was intended to study the changes in the ground substance and the role of growth processes in the articular cartilage during the early stages of the disease. Growth processes in osteoarthritis are usually interpreted as reactive or as attempts at regeneration.[2,3] There seems to be little doubt that this concept applies to growth occurring during advanced phases of the disorder, but it does not necessarily apply to early growth processes preceding the development of osteoarthritis in the strict sense.[5-9] In view of the role generally attributed to the trauma of weight-bearing, it was considered advisable to study a non-weight-bearing articulation such as the sternoclavicular joint. In most investigations concerned with this articulation,[6,10-15] microscopic details were not systematically dealt with, and histochemical techniques were not applied at all. The use of such methods, however, seemed essential for the understanding of the sequence of events leading to osteoarthritis. The available material also gave an opportunity to evaluate the relationship of the joint disease to such metabolic disorders as arteriosclerosis, obesity and diabetes.

MATERIAL AND METHODS

Sternoclavicular joints were obtained from 200 individuals (100 males and 100 females) dying at the St. Louis City, Missouri Pacific, and Barnes Hospitals. The joints of at least 10 males and 10 females were collected in each decade; the groups including the second and third decades were smaller since mortality at this age was low and material therefore difficult to secure.

Occupation, state of nutrition, main disease, and cause of death were recorded with special reference to the existence of metabolic disorder

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and to possible trauma to the joint. Corresponding to that of the hospital population, the racial distribution was 84 per cent white and 16 per cent Negro. Most individuals were public welfare patients with comparatively low living and housing standards; a few were skilled laborers or white collar workers. The adult groups were composed of about equal numbers of employed, unemployed, and retired individuals. Women were usually housewives or engaged in light manual work.

The distribution of the patients according to sex, race, age, and causes of death is indicated in Tables I to III.

At necropsy the sternoclavicular joint of the right side, but occasionally that of the left, was removed, including portions of the sternum and clavicle. The tissue blocks were frozen, sawed in two with an electric saw, thawed, fixed in 10 per cent formalin, and decalcified in 5 per cent nitric acid. The tissue was then trimmed, transferred to 2 per cent potassium alum for 24 hours and washed in running water for 48 hours. Paraffin or a combination of paraffin and celloidin was used for embedding. The blocks were sectioned at different levels and stained with hematoxylin and eosin, with toluidine blue for metachromasia, or with the Gomori trichrome and periodic acid-Schiff (PAS) stains for mucopolysaccharides. The various features observed were graded according to criteria given below, and the data obtained were, whenever possible, analyzed statistically by the Chi square method.

Observations

The histologic features of the normal sternoclavicular joint have been fully described.[10,12,14,15] According to criteria which we have used previously and described in detail,[4] age changes may as a rule be distinguished from osteoarthritis. Briefly, alterations attributable to age, were represented by hyperplasia, hypertrophy and degeneration of cartilage, as long as the articular surfaces and the discs were intact, and as long as there was no vascularization of the cartilage. Although these alterations usually increase with advancing age, they may, even in very old individuals, remain stationary for a considerable length of time. Osteoarthritis was of hypertrophic or of ulcerative type; it was characterized by vascularization, fraying, and overgrowth of cartilage with formation of villi and loose bodies; or by deep ulcerations with ossification, and fibrosis and cyst formation of the adjacent bone marrow. Osteoarthritis was considered as mild if the defects of the articular surfaces and discs were represented by focal erosions, and if no marginal growth was observed; otherwise, the lesions were designated as severe.

Results

First Decade (20 cases). The articular cartilage consisted of several surface layers of primitive cartilage cells embedded in fibrillar ground substance. Below these layers, young cartilage cells similar to those found in hyaline cartilage were arranged in typical columns undergoing endochondral ossification, and were delimited from the bone marrow by a thin, discontinuous layer of bone. The matrix was homogeneous, and gave a weakly positive PAS reaction (Fig. 1). The

Table I

Articular Alterations in 100 Sternoclavicular Joints in Human Males and Their Distribution According to Age and Race

Decade	Race	Mean age (yrs.)	No. of patients	No alterations no.	Age alterations no.	Osteoarthritis		
						Slight no.	Advanced no.	Total no.
1	White	3.5	8	8	0	0	0	0
	Negro	3	2	2	0	0	0	0
2	White	14.5	4	4	0	0	0	0
	Negro	15	1	1	0	0	0	0
3	White	24	5	3	2	0	0	0
	Negro	27	3	1	2	0	0	0
4	White	35	9	2	6	1	0	1
	Negro	33.5	2	0	1	1	0	1
5	White	47	10	1	3	3	3	6
	Negro	—	0	—	—	—	—	—
6	White	55.5	11	0	1	6	4	10
	Negro	51	1	0	0	0	1	1
7	White	67	10	0	1	3	6	9
	Negro	—	0	—	—	—	—	—
8	White	75.25	11	0	1	1	9	10
	Negro	79	1	0	0	0	1	1
9	White	84.25	7	0	1	0	6	6
	Negro	84	3	0	0	1	2	3
10	White	94	12	0	1	7	4	11
	Negro	—	0	—	—	—	—	—
Subtotal	White	57.5	87	18	16	21	32	53
	Negro	42.25	13	4	3	2	4	6
Total		55.25	100	22	19	23	36	59

discs were composed of spindle-shaped cells separated from each other by dense, eosinophilic, delicately metachromatic, and slightly PAS-positive material. Sex differences in the progress of endochondral ossification could not be established.

Second Decade (14 cases). The articular surfaces were smooth and

showed the usual wavy indentations. The cartilage cell columns were short; toward the end of the decade the cells of the deeper layer formed an increasing number of isogenous groups ("territories"). Growth of cells and endochondral ossification slowed down and came to a standstill. The matrix increased in amount, and metachromasia increased as one proceeded from the resting toward the hypertrophic

TABLE II

Articular Alterations in 100 Sternoclavicular Joints in Human Females and Their Distribution According to Age and Race

Decade	Race	Mean age (yrs.)	No. of patients	No alterations no.	Age alterations no.	Osteoarthritis		
						Slight no.	Advanced no.	Total no.
1	White	4	8	8	0	0	0	0
	Negro	4.25	2	2	0	0	0	0
2	White	13.5	5	5	0	0	0	0
	Negro	14.25	4	4	0	0	0	0
3	White	26	6	2	4	0	0	0
	Negro	27	3	1	1	1	0	1
4	White	34.5	9	0	7	2	0	2
	Negro	35	2	0	0	2	0	2
5	White	45	7	0	2	3	2	5
	Negro	42	3	0	0	0	3	3
6	White	56	9	0	3	5	1	6
	Negro	54	2	0	0	1	1	2
7	White	65.5	7	0	0	5	2	7
	Negro	63.25	3	0	0	0	3	3
8	White	73	10	0	0	4	6	10
	Negro	—	0	—	—	—	—	—
9	White	84	10	0	1	4	5	9
	Negro	—	0	—	—	—	—	—
10	White	92.25	10	0	0	8	2	10
	Negro	—	0	—	—	—	—	—
Subtotal	White	42.25	81	15	17	31	18	49
	Negro	32.25	19	7	1	4	7	11
Total		40.5	100	22	18	35	25	60

ossifying cartilage (Fig. 4); more PAS-positive material was seen than earlier in life (Fig. 6). The presence of many collagen fibers now gave the cartilage the appearance of typical fibrocartilage. A continuous, thick, transverse, bony lamella delimited the quiescent cartilage from the bone marrow. The trabeculae were thicker and coarser than before. The cells of the disc became vacuolated, and the cell capsules thick-

ened, while much PAS-positive material had been laid down in the intercellular matrix. From the age of 12 years on, the development of the female joint was farther advanced than that of males. This was indicated by relatively decreased cell proliferation and relatively advanced ossification of the cartilage of the former.

Third and Fourth Decades (39 cases). These two decades are discussed together because they have in common the first appearance of age alterations as well as of osteoarthritis. From the early part of the third decade on, the growth zones were closed. The cell territories of the intermediate layer and those close to the zone of replacement gradually became more cellular, and the cells underwent hypertrophy. These *"Brut"* capsules were surrounded by basophilic halos. Subsequently a number of the cells degenerated, and faded (*"Verdämmerung"*) into the surrounding matrix. The changes proceeded from the deeper layers toward the surface and ultimately reached the latter. The matrix was swollen, loosened, and had a peculiar bluish-gray quality with the hematoxylin and eosin stain; finely granular matter became visible. There was comparatively little metachromasia (Fig. 5), but abundant PAS-positive material was manifest (Fig. 2). On the clavicular surface the alterations set in earlier and were more accentuated than on the sternal aspect of the joint. The discs likewise showed hyperplasia, hypertrophy and degeneration of cells, and in the matrix large patches of PAS-positive substance were identified.

These age changes were occasionally superseded by early osteoarthritis, as indicated by the presence of clusters of proliferating, hypertrophic cartilage cells, which showed little calcification, but were eroded by capillaries advancing from the bone marrow. In such areas of growth the matrix was metachromatic, and the PAS reaction was weakly positive or negative (Fig. 7). Subsequently, both growth and regressive processes were intensified and involved the articular surfaces and the disc. Of 39 cases comprising these 2 decades, 9 joints (23 per cent) were unaltered, 23 joints (59 per cent) showed age changes, and 7 joints (18 per cent) disclosed mild osteoarthritis. Severe lesions were not seen.

Fifth and Sixth Decades (43 cases). During this period of life the incidence of osteoarthritis increased considerably, and the lesions tended to be severe. This was indicated by deformity, the formation of villi and loose bodies, and by partial or complete obliteration of the joint cavity due to adhesions between the synovial, disc and articular surfaces. The matrix of the cartilage showed different degrees of meta-

chromasia and varying amounts of PAS-positive material. If growth of cartilage was marked, the matrix became intensely metachromatic and contained much PAS-positive substance, while quiescence of cells was characterized by little if any metachromasia. The amount of PAS-positive material depended on whether or not the cartilage was undergoing ossification. Ossifying cartilage contained much PAS-positive substance, but non-ossifying cartilage was only slightly or not at all PAS-positive (Figs. 3 and 8).

Among all joints in these groups, only one (2 per cent) was unaltered—that of a 48-year-old white male who met death accidentally. Nine joints (21 per cent) showed age alterations, but 14 cases of osteoarthritis (70 per cent) were found in the fifth, and 19 cases (83 per cent) during the sixth decade. Of the 33 arthritic patients, 15 joints (45 per cent) showed severe forms of the disorder.

Seventh to Ninth Decades (62 cases). The severity of arthritis was further intensified, and the incidence of the lesions reached its peak. From the seventh decade on, there were no normal joints, and only a rare articulation (6 per cent) showed advanced age changes, while 58 joints (94 per cent) disclosed osteoarthritis. Forty of these lesions (69 per cent) were severe.

Tenth Decade (22 cases). Of the individuals surviving into the tenth decade, a single male, 92 years old, showed marked age changes of the joint; the joints of all others (95 per cent) were the seat of osteoarthritis. However, only 6 of the 21 arthritic joints (28 per cent) had advanced lesions. This represents a highly significant decrease in the severity of the disorder ($P<.001$). In these old patients one is apparently dealing with a selected population. These individuals reach old age because their physiologic rate of aging is slow, and therefore, presumably, their tissues also age slowly. The slow rate of aging of the articular tissues in turn results in delayed development of osteoarthritis.[5]

Significance of Sex and Race

Sex. The early retardation of maturation of the joints of males was offset by accelerated aging later in life. The ultimate incidence of arthritis was similar in both sexes; however, in males, the lesions were more severe than in females. Thirty-two of 53 arthritic joints (60 per cent) in white males had advanced lesions, while in 49 white females with arthritis, only 18 joints (37 per cent) were severely affected. This difference is significant at the 5 per cent level and almost significant at the 2 per cent level; it could not have been in-

fluenced by age, because the mean ages of both groups were similar (65.75 years for males and 65.5 years for females).

Race. The number of Negroes included in this series was too small to permit statistical analysis of the data obtained. However, certain trends may be recognized. A higher susceptibility to arthritis in Negroes as compared to whites seems suggested by the considerably lower age (28.5 years as compared to 41.3 years) at which articular age changes were seen in the former. The same trend manifested itself also in the low mean age at which arthritis was found in Negro females. In the latter the total incidence (59 per cent) was about the same as in white women (60 per cent). However, in Negro women, this incidence was already reached at a mean age of 32.25 years. At a comparable age arthritis was found in only 22 per cent of white women. In the latter the maximum incidence was not reached before a mean age of 41.75 years.

Significance of Metabolic Disorders

It has been pointed out repeatedly that there might be some relationship between osteoarthritis on the one hand and arteriosclerosis, obesity, and diabetes on the other. In the following paragraphs, the available data are evaluated in this respect.

Arteriosclerosis. Since both arteriosclerosis and arthritis are frequent during the later periods of life, one has to expect a certain concordance of the incidences of these conditions on merely mathematical grounds. This, however, need not indicate a biologic relationship. Of the 166 patients comprising the third to the tenth decades, 55 per cent had mild and 17 per cent had severe arteriosclerosis. Of these 166 individuals, 118 showed arthritic lesions, and of those who had arthritis, 66 per cent disclosed mild and 25 per cent severe arteriosclerosis. There was a 50 per cent concordance, inasmuch as 30 patients (18 per cent) had neither arteriosclerosis nor arthritis, and 52 individuals (32 per cent) had both. The remaining 84 patients (50 per cent) had either arthritis or arteriosclerosis. This distribution indicates a lack of correlation between the two diseases. Other comparisons relating to the severity of the lesions and to the ages at which both diseases occurred had the same negative results.

Obesity. There was one woman 29 years of age and weighing 600 lbs., whose joint showed advanced age alterations but no osteoarthritis. Of the 118 individuals with arthritis, 20 were obese at the time of death, and 10 others, it was learned from the records,

had been obese for some time prior to death. Among the 30 obese patients, 12 were males and 18 were females. Thus, so far as could be ascertained, one fourth of the individuals with arthritis were also obese at one time or another, and 60 per cent of them had arthritis of severe degree. On the other hand, there was no record of obesity in the vast majority of patients with arthritis; there was thus little evidence in favor of a primary role of obesity in the pathogenesis of osteoarthritis. However, it may be significant that 17 of the 30 obese individuals with arthritis were only 34 to 48 years old. In the unselected population, the incidence of arthritis during the fourth and fifth decades ranged between 30 and 40 per cent; no severe cases were found during the fourth decade, and only a few during the fifth decade. Therefore, the especially high incidence of severe lesions in comparatively young obese individuals indicates a possible relationship between obesity and osteoarthritis. The nature of this relationship is not known. However, since a non-weight-bearing joint is concerned, the link between osteoarthritis and obesity may be represented by metabolic factors rather than by the mechanical stress of increased weight-bearing.

Diabetes. The present series included 6 diabetic patients: 4 white females, and 1 white and 1 Negro male respectively. All 6 had severe osteoarthritis. Since in unselected white females the joint disorder had a comparatively slow course, the existence of severe arthritis in all 4 diabetic females at the comparatively low mean age of 46 years may be more than a coincidence.

Relationship Between Osteoarthritis and Causes of Death

In Table III, the causes of death of 166 patients dying at ages over 20 years are summarized. The corresponding data for the 118 patients with arthritis are given in the second horizontal column. The causes of death are classified according to the following major

TABLE III

Causes of Death in Patients Dying after the Age of 20 Years

Group	No.	Causes of death						
		Cardio-vascular	Pulmo-nary	Gastro-intestinal	Renal	Malignant tumors	Acci-dents	Misc.
Total population	166	71	30	12	10	23	10	10
Arthritic population	118	53	27	3	7	19	5	4

groups: (a) cardiovascular diseases, including arteriosclerosis, hypertension, coronary and cerebral thrombosis; (b) pulmonary diseases, including pneumonia, tuberculosis, asthma, emphysema and bronchiectasis; (c) diseases of the digestive system, such as peptic ulcer, biliary disease, hepatitis, and cirrhosis; (d) renal diseases, including polycystic kidney and nephrolithiasis; (e) miscellaneous disorders such as chronic nervous and muscle diseases, epilepsy, diabetes, and blood dyscrasias; (f) accidents; and (g) malignant neoplasms.

Few arthritic patients died of gastrointestinal diseases or met an accidental death. This may be due to the fact that patients dying from these causes were comparatively young and had not yet reached the arthritis age. All patients dying of chronic kidney disease had severe osteoarthritis although their mean age at death was only 47 years. Otherwise, there was a lack of correlation between the causes of death and the presence or absence of arthritis. Regardless of the cause of death, the sternoclavicular joint may be affected in a number of generalized disorders.[14] In our cases there were 3 instances of rheumatoid arthritis and one of Marfan's syndrome with severe alterations in the joints.

Discussion

Examination of 200 human sternoclavicular joints disclosed age alterations as early as the third decade of life. These observations agree with those previously recorded in this and other articulations.[3,10,14] With advancing age of the individuals, the alterations became more severe and progressed into osteoarthritic lesions.[3,4] Ultimately, over 90 per cent of the joints were affected. The involvement of the sternoclavicular joint in osteoarthritis was thus by no means as uncommon[10,12,15] as would appear from clinical and some pathologic investigations.[6] In agreement with a previous observation,[11] the interarticular discs and clavicular cartilages were affected earlier, more frequently, and more severely than the sternal surfaces. In young females maturation of articular cartilage proceeded at a faster rate than in males. With advancing age, however, this temporary delay was compensated for by an accelerated rate of aging, and a more marked severity of joint lesions in males. These findings correlate well with those established for the knee joints of humans[3] and laboratory animals.[4]

Whether or not the more severe course of degenerative joint disease observed in Negroes as compared with whites holds good in general,

will have to be substantiated in a larger series of individuals. It is possible that such differences exist. Differences in the susceptibility of various strains of mice to degenerative joint disease have been established.[4,16,17]

The coexistence of atherosclerosis and osteoarthritis appears to be merely coincidental. The occurrence of severe osteoarthritis in comparatively young individuals with diabetes, on the other hand, may be significant. This relationship is presently under investigation. The possible significance of chronic renal disease in the development of osteoarthritis, to which apparently not much attention has been paid will likewise require exploration. In both of these conditions hormonal imbalances exist which may possibly act as mediators in the development of severe joint disease, since hormones exert profound influences on articular cartilage.[5]

Obesity was often associated with osteoarthritis, and there was a striking coexistence of severe osteoarthritis and obesity in comparatively young individuals. However, the majority of individuals with osteoarthritis had no history of obesity. These observations are in agreement with those made in animals,[5] and would speak against a primary role of overweight in the pathogenesis of osteoarthritis. If there is any link between obesity and osteoarthritis, the findings in the non-weight-bearing sternoclavicular joint would indicate that this relationship rests on conditions other than mechanical factors.

The earliest abnormalities related to age were hyperplasia and hypertrophy of cartilage cells. Such growth processes are not peculiar to aging articular cartilage, but have also been observed in aging cartilage of rib and trachea.[5,7] They have been attributed variously to functional stress,[18] reaction to injury caused by wear and tear,[3] or disturbed vascularization.[8] Since growth of cartilage was diffuse in the articular covering and in areas exposed to pressure, as well as in those not subjected to pressure,[8,10] it seems unwarranted to consider functional stress as a cause. Certainly there exists no stress of this kind in aging costal and tracheal cartilages. Moreover, extraskeletal tissues such as skin, thyroid, prostate, and endometrium may likewise undergo primary growth stimulation during early phases of aging.[19,20]

In aging joints, early growth was associated with or preceded by alterations in metachromasia and loss of PAS staining in the matrix. These alterations in ground substance may be interpreted as a change from a relatively depolymerized state to one of higher poly-

merization. An alteration in polymerization, however, would hardly represent a process of degeneration in the strict sense. Cartilage has been shown to react promptly to experimentally induced changes in the internal environment.[5] The same may be true for physiologic fluctuations in this environment as they are apt to occur during the life span of the individual. Conceivably then, the cartilage undergoes periodic changes; some of these may concern cells primarily; others may primarily affect the matrix, which changes its state of polymerization. As a consequence, the matrix might lose some of the restraining powers which it usually exerts on cell growth, and hypertrophy and hyperplasia of cartilage cells would thus be resumed. Such release of growth would then not represent a reaction to injury. Once there is excessive growth of cartilage, the further evolution of osteoarthritis is readily understood. The new cartilage cannot be adequately maintained, but undergoes degeneration. The ensuing reactive growth is followed by further regression, until the latter becomes the prominent feature. In the further development of osteoarthritis the role of vascularization of cartilage is well established, although the mechanisms which lead to this resumption of endochondral ossification—possibly a frustrated attempt at regeneration—are not understood.[8] The present observations thus indicate the need for further biochemical and biophysical analyses of aging cartilage.

Summary

Sternoclavicular joints of 200 individuals ranging in age from the first to the tenth decades were studied for the existence of age alterations and osteoarthritis, with particular reference to the role of early growth processes. Joint lesions of both types appeared during the third decade. The incidence and severity of osteoarthritis increased up to the age of 80 years. In individuals over 90 years of age, the incidence of severe arthritis was strikingly decreased. The lesions found in males were more severe than in females, and Negroes seemed to be more susceptible than whites. There was a positive correlation between osteoarthritis and diabetes and chronic renal disease, and between severe osteoarthritis and obesity; however, no such correlation was found to exist between osteoarthritis and arteriosclerosis. The relationship between arthritis and obesity does not seem to be based on mechanical factors.

Hyperplasia and hypertrophy of the articular cartilage cells occurred early in the aging process and were associated with changes in meta-

chromasia and loss of PAS-positive staining in the matrix. These biochemical and biophysical changes in the intercellular substance are thought to interfere with the restraining influence usually exerted by the matrix on cell growth. As a consequence, the dormant growth potential of the cartilage cells may be released, and the ensuing growth may set off the chain of events which lead to the complex structural lesion of osteoarthritis.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

Sections of the clavicular aspect of the sternoclavicular joint.

FIG. 1. Four-week-old white boy. Growth of cells and endochondral ossification in progress. No PAS-positive material seen. Periodic acid-Schiff stain. × 27.

FIG. 2. Thirty-five-year-old white male. PAS-positive material abundant in ossifying cartilage, scanty between hyperplastic and hypertrophic cartilage which forms "territories." Periodic acid-Schiff stain. × 27.

FIG. 3. Ninety-two-year-old white male. Advanced osteoarthritis with active villous growths. Little or no PAS-positive material in these areas. Periodic acid-Schiff stain. × 27.

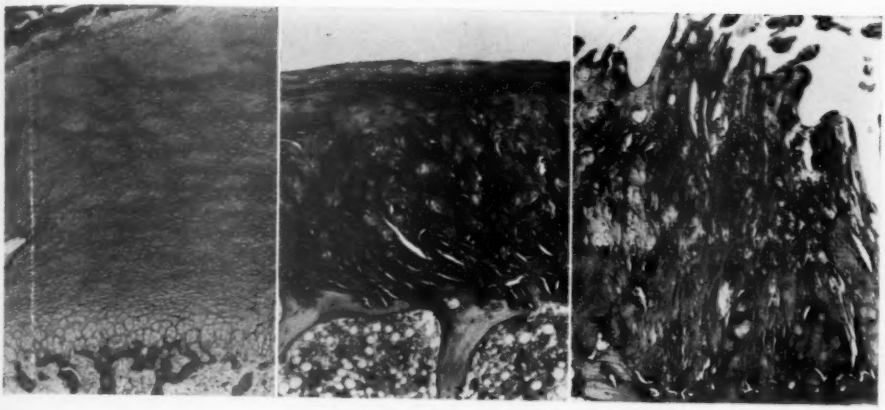
FIG. 4. Twenty-three-year-old Negro female. Endochondral ossification has ceased. Formation of "territories" in intermediate and deep zones with metachromasia of matrix. Toluidine blue stain. × 40.

FIG. 5. Thirty-six-year-old white female. Little metachromasia in the deep layers of proliferating cartilage. Early osteoarthritic change at the surface. Toluidine blue stain. × 40.

FIG. 6. Twenty-five-year-old white female. Cartilage contains PAS-positive material which increases in amount as one proceeds from the surface to the deeper layers. Periodic acid-Schiff stain. × 27.

FIG. 7. Thirty-nine-year-old white female. Focus of proliferation of cartilage near area of ossification. PAS-positive material disappeared from areas where growth of cartilage is resumed. Early osteoarthritis. Periodic acid-Schiff stain. × 27.

FIG. 8. Eighty-two-year-old white female. Severe osteoarthritis with obliteration of the joint cavity. Less growth and more PAS-positive material than in Figure 3. Periodic acid-Schiff stain. × 27.



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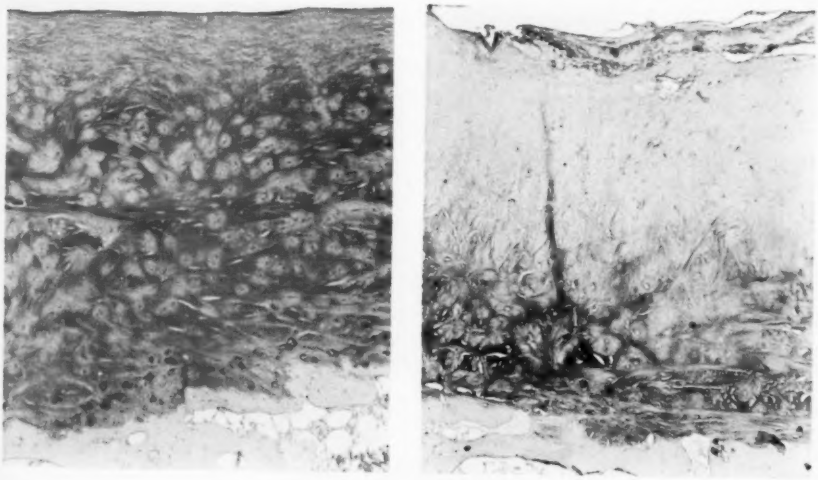

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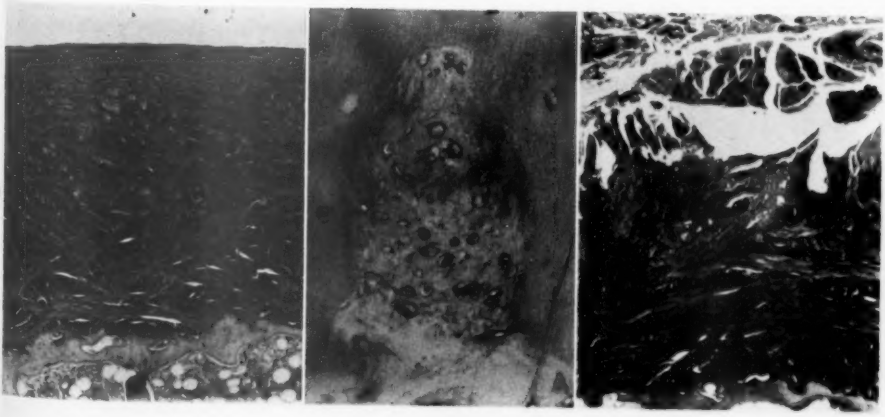

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CORONARY ATHEROMATOUS CHANGE INDUCED BY CHRONIC HYPERCHOLESTEROLEMIA IN DOGS*

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The effect of induced chronic hypercholesterolemia on canine aortic transplants has been investigated in this laboratory for the past few years.[1-3] During the course of these studies, the development of atheromatous lesions was noted in other areas than in the graft, particularly in the coronary arteries. In canine vessels[4] the production of atheromatous lesions has been accomplished somewhat less readily than in vessels of other animals, such as the rabbit.[5] In this paper the observations on coronary atheromatous lesions induced in the dog are recorded.

MATERIAL AND METHODS

The production of chronic hypercholesterolemia was attempted in 84 dogs by thyroid ablation, using radioactive iodine, and by cholesterol administration in the diet, as previously reported.[1] All of these animals had grafts or synthetic prostheses placed in various portions of the aorta. At the termination of each experiment the heart was preserved in 10 per cent formalin, and the presence or absence of gross lesions in the coronary arteries were noted for comparison with lesions at other sites. For the present study, the hearts with atheromatous lesions were cut either in a frontal plane or perpendicular to the longitudinal axis, and representative portions of the ventricles, including the anterior descending branch of the left coronary artery, were taken for microscopic examination.

Paraffin sections were cut and stained with hematoxylin and eosin and by Masson's trichrome and Weigert's elastic tissue stains. In addition, from some of the affected coronary arteries, frozen sections were prepared and stained with Scharlach R.

RESULTS

Of the 84 dogs used, 39 developed atheromatous lesions in the aorta. Among these, 13, both male and female animals, disclosed gross and microscopic atheromatous lesions in the coronary arteries.

The shortest period of cholesterol administration prior to the de-

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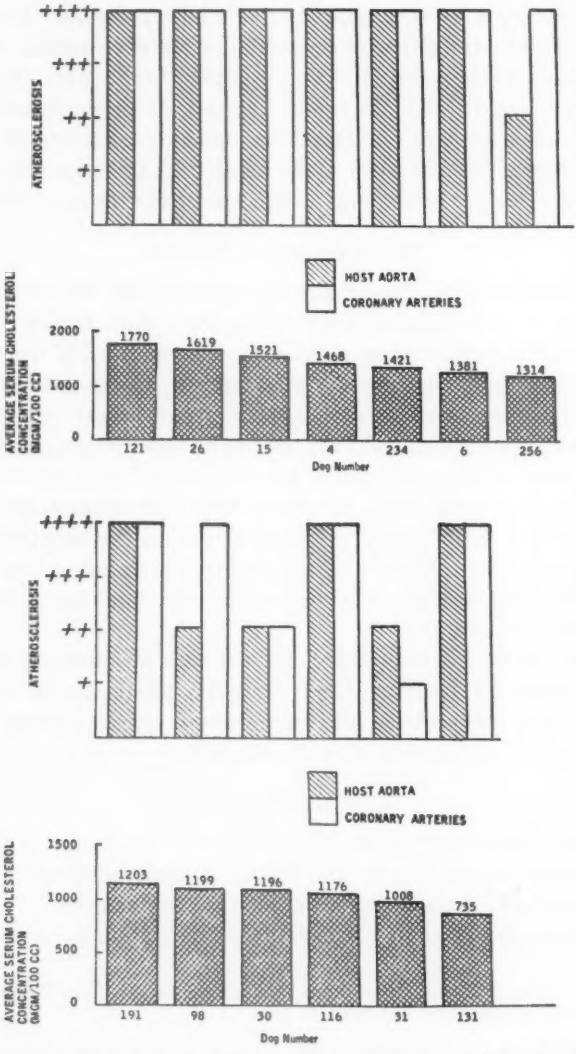
velopment of gross atheromatous lesions in the coronary arteries was 5 months, and the longest 14 months; the average was 7.4 months. The average serum cholesterol concentrations maintained were between 735 and 1,770 mg. per cent. The average for all animals was 1,309 mg. per cent. In only one animal was the average serum cholesterol level less than 1,000 mg. per cent (Text-fig. 1).



Text-figure 1. Average serum cholesterol concentrations and induced atheromatous alterations in the coronary arteries and aortas in 13 dogs.

Grossly, the involved coronary arteries and their branches were thickened, tortuous, beaded and yellow (Figs. 1, 3 and 5).

Microscopically, large foam cells contained a lipid substance, mostly isotropic, replacing extensive portions of the intima and media. The lipid-laden cells were distributed along the endothelial lining of the *vasa vasorum* but were also noted elsewhere, broadening the intima, narrowing the lumen, and replacing large parts of the media with interruption of the inner elastic lamina. The lesion usually extended about the entire circumference of the vessel wall. Areas of atheromatous alteration, with hemorrhage, lipid debris, and slitlike spaces, were noted in scattered fashion (Figs. 2, 4 and 6). There was a striking resemblance of the lesions to those observed in man, but they were more diffuse. Though the intimal and medial alterations in the coronary arteries were extensive, obviously encroaching upon the caliber of the lumen, in no instance was there any demonstrable infarction of the myocardium.

Comment

The maintenance of high serum cholesterol concentrations for prolonged periods was the primary requisite for the development of coronary atheromatous lesions in these animals. Only one animal (# 131) with an average serum cholesterol concentration of less than 1,000 mg. per cent developed these lesions. That animal was on the diet for the longest period, 60 weeks.

In the dog the coronary arteries are less prone to develop atheromatous alteration than the abdominal aorta. However, the lesions in the coronary arteries were often more extensive than those in the thoracic aorta.

It is of special interest that none of the 13 animals with extensive coronary involvement died of myocardial infarction. However, the animals led a relatively sedentary life during the period of the experiment with regular exercise but were not subjected to stressful situations. The coronary atheromatous alteration induced in the dog, with its close resemblance to the lesions observed in the human, permits the study of the natural history of the lesion.

Summary

Coronary atheromatous lesions were induced in 13 of 84 dogs subjected to thyroid ablation by radioactive iodine and fed a high cholesterol diet. The alterations produced closely resembled those of coronary atheroma in man. However, none of the animals developed infarction of the myocardium. The method provides a means for the study of the natural history of the lesion.

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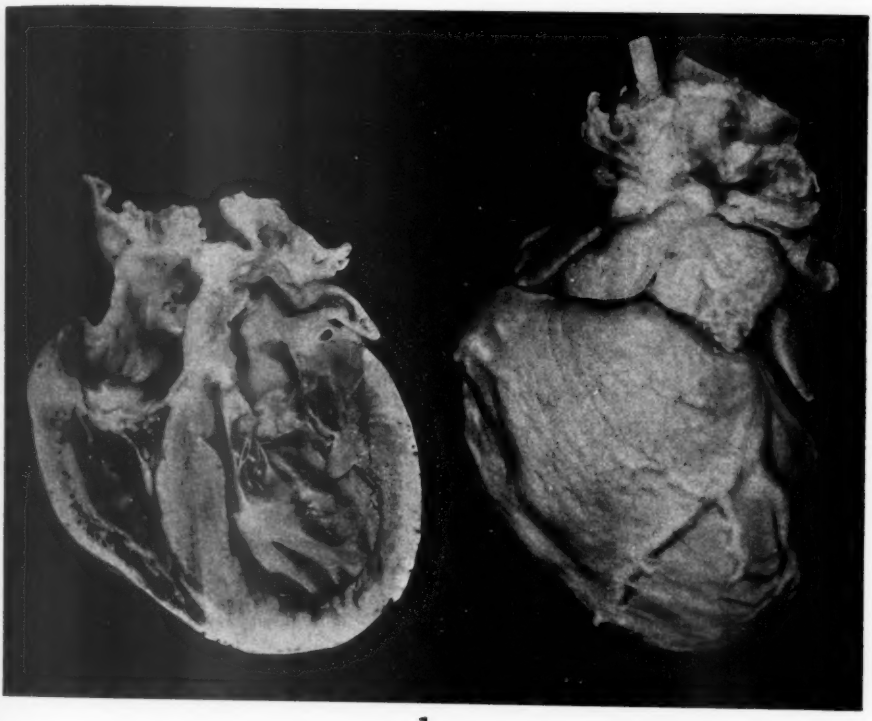
LEGENDS FOR FIGURES

FIG. 1. Gross appearance of atheroma formation in the coronary arteries of dog 98. The anterior descending branch and its subdivisions are clearly outlined. Thickening of the wall of the sectioned vessel and narrowing of its lumen by atheroma are evident.

FIG. 2. The wall of a twig of the anterior descending branch of the left coronary artery shown in Figure 1 is almost completely replaced by large foam cells. These vary in size and have small eccentrically placed nuclei. In other fields lipid debris is in vacuoles and elsewhere slitlike spaces are numerous. Masson's trichrome stain. × 80.



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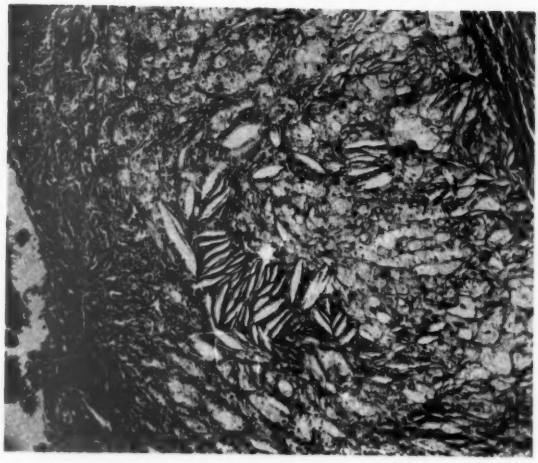

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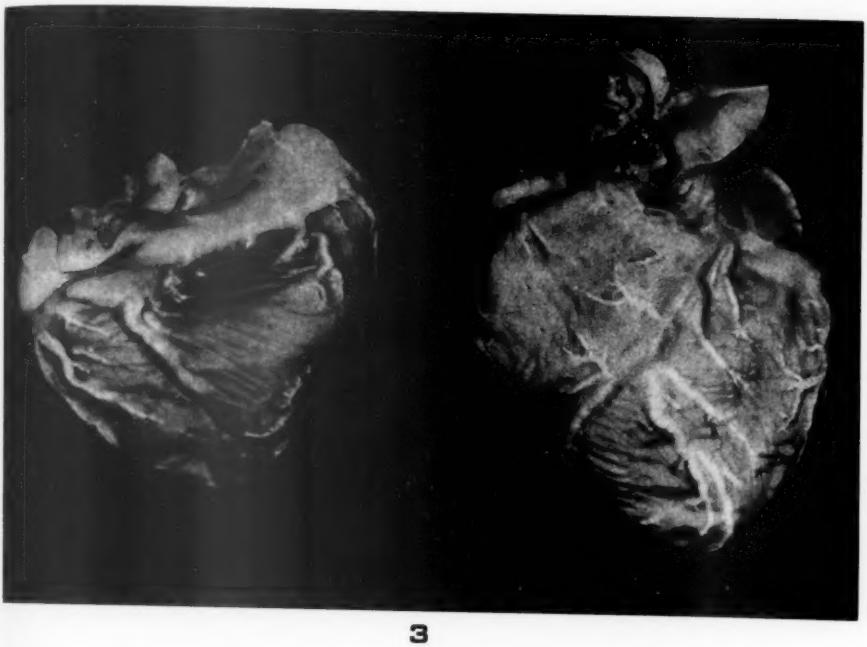
FIG. 3. Gross appearance of atheroma formation in the coronary arteries of dog 121. The coronary arteries and their branches are elevated above the surface and are beaded and tortuous.

FIG. 4. Microscopic appearance of the wall of a small branch of the left coronary artery from the heart shown in Figure 3. The lumen is markedly reduced by lipid-containing foam cells and debris. These replace practically all of the media about the entire circumference of the wall. Masson's trichrome stain. × 100.



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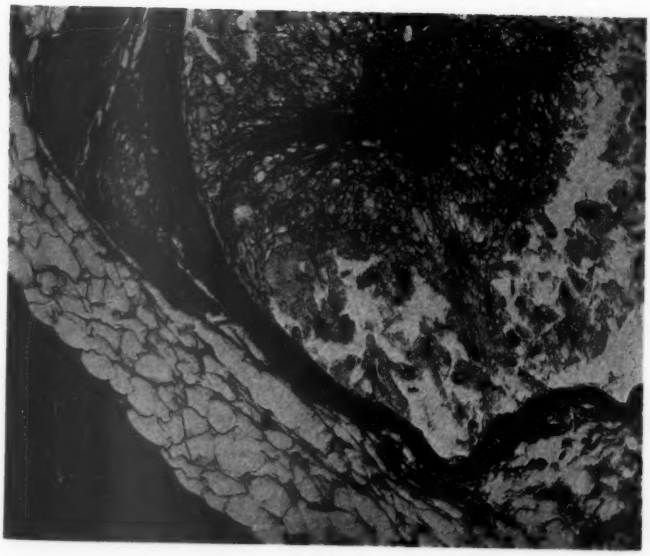

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FIG. 5. Gross appearance of atheroma formation in the coronary arteries of dog 131. The arteries and their branches are tortuous and beaded.

FIG. 6. Microscopically, the lumen of the anterior descending branch of the left coronary artery shown in Figure 5 is widely patent. A frayed inner elastic lamina marks the border between the intima and media. Lipid-laden foam cells almost completely replace the media and broaden the intima. Weigert's elastic tissue stain. × 100.

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6

ALTERATION OF STRAIN SPECIFICITY OF SARCOMA I IN ITS NATIVE A STRAIN HOST:

Effect of Immunization with Normal and Cancerous Tissues *

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There have been numerous investigations of induced or acquired tolerance in relation to homotransplants of both cancer and normal tissue. The most frequent approach has been to treat the recipient animal pre- or postnatally by various means, e.g., whole tissue homogenates, extracts of normal and cancerous tissues and their respective antiserums, lymph node substance from immunized animals, cell fractions, cortisone and x-irradiation.[1-10] Hašek has reported another approach to induction of homograft tolerance utilizing embryonic parabiosis in birds. This involves mutual influences between donor and recipient as parabionts.[11]

In earlier studies, we showed that certain tumors could be successfully transplanted to mice of different strains if the recipients were pretreated with lyophilized tissues from the donor strain. It was also demonstrated that the administration of cortisone with the lyophilized tissue led to a metastatic spread of the transplanted tumor.[12-14] Moreover, it appeared that successfully transplanted tumors underwent changes in strain specificity.[15] These studies, combined with additional data[16,17] on mouse tumors, and Greene's observations[18,19] concerning heterotransplantability of human tumors, have led to the hypothesis that successful homotransplantation requires adaptation both of host to tumor and of tumor to host.

The present investigation deals with an effort to secure successful homotransplantation of a tumor to an otherwise incompatible recipient by adapting the transplant *in vivo* prior to its excision from the donor. Recipient animals were not treated in advance of inoculation.

Material and Methods

The tumor used was Sarcoma I which originated at the University of California in 1947. We obtained it from the Jackson Laboratories,

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Bar Harbor, Maine, in 1951, in its 152nd transplant passage. Since then it has been carried continuously in A/He strain mice in our mouse colony. As is well known, Sarcoma I grows equally well in A/Jax mice and in 100 per cent of F-1 hybrids, both male and female. On the other hand, it is ordinarily rejected by other conventional laboratory strains of mice such as C3H, C57BL/6, and BALB/c. Following successful transplantation, the tumor becomes palpable within 2 to 3 days, and death occurs within 3 weeks. The lesion is characterized by dense sheets of spindle-shaped and polyhedral cells, the latter predominating. Mitotic figures are numerous and stroma is scanty.

In our laboratory, in the course of transplantation of Sarcoma I to A/He and A/Jax mice, we have also routinely introduced the tumor into C3H, BALB/c and C57BL/6 mice after every tenth to twelfth passage in A strain mice. Since 1951, in no instance has the Sarcoma I survived or grown in any of the outstrain passages.

Two groups of 20 normal adult A strain mice, 10 to 12 weeks of age, each comprised of 10 males and 10 females, were treated with lyophilized tissue from C57BL/6 donors. Each mouse received 45 mg. of the inoculum as follows:

Group I. Each mouse received 3 15-mg. injections of mixed lyophilized liver, spleen and kidney (equal parts by weight). The inoculum was homogenized in 0.5 ml. of buffered saline (0.85 per cent NaCl) and injected intraperitoneally at 5-day intervals.

Group II. Each mouse received 3 15-mg. injections of lyophilized tumor EO771, an adenocarcinoma of mammary gland, harvested from C57BL/6 mice. The total dose by weight, interval and route of injection was the same as in Group I.

Adenocarcinoma EO771 is a spontaneous tumor of the mammary gland recovered in 1939 from a C57BL/6 mouse. This tumor is species specific and normally will not grow in any other strain. We obtained it from the Jackson Laboratory in 1953 and have carried it since that time in a C57BL/6 line of mice bred in our mouse colony. The tumor "takes" 100 per cent in C57BL/6 mice, is palpable in 3 to 5 days and kills in 19 to 56 days. It rarely metastasizes, but invariably undergoes extensive necrosis. Filtrate extracts of the tumor are not transmissible.

Ten days after the last injection in the above groups, each of the treated A strain mice was grafted with a pooled mixture of live Sarcoma I (approximately 1 cmm. in size). The tumor was obtained from 6 tumor-bearing A strain donors. Implantation was made subcutaneously in the nuchal region with a 5-inch, 15-gauge trocar inserted near the tail and passed subcutaneously along the animal's body to the site of

implantation. All tumor implantations were made under rigidly aseptic conditions. Cultures in thioglycollate media were made of all tumor minces as well as of the lyophilized tissue in order to assure freedom from bacterial contamination.

Attention is specifically directed to the fact that the Sarcoma I graft is "native" to the A strain mice and is carried routinely in this strain. Simultaneously with the implantation of a viable graft in the treated A strain mice, Sarcoma I tumor was also implanted in 10 normal adult mice in each of A/Jax, C3H, C57BL/6 and BALB/c strains. The tumor was palpable by the fourth day in both the treated and untreated A/Jax mice and achieved a size greater than 1.5 cm. by the tenth day. The implant did not survive and grow in any of the C3H, C57BL/6 or BALB/c control mice during a 60-day period of observation.

When the tumor in the treated A strain mice had achieved a size of 1.5 cm. to 2 cm. in a period of 7 to 9 days, 5 mice from each of the 2 treated groups (i.e., group I, those treated with lyophilized normal C57BL/6 tissue; and group II, those treated with lyophilized EO771 tumor tissue) were sacrificed on the ninth day after implantation of the live Sarcoma I tissue. The tumor was removed aseptically and transplanted directly, by trocar, in accordance with the technique previously described, into the following normal adult mice:

Sarcoma I from A strain mice treated with normal C57BL/6 tissue (designated as Sa I/Wald 2): Transplanted to 18 C3H mice; 19 BALB/c mice; 20 C57BL/6 mice; 10 A/Jax mice.

Sarcoma I from A strain mice treated with lyophilized EO771 tumor (designated as Sa I/Wald 3): Transplanted to 18 C3H mice; 18 BALB/c mice; 20 C57BL/6 mice; 10 A/Jax mice.

The graft was examined and identified in every passage by microscopic examination of formalin fixed tissues in hematoxylin and eosin stained sections.

Results

In the case of both Sa I/Wald 2 and Sa I/Wald 3, the implants grew in all 10 of each group of A/Jax mice in a fashion similar to the unchanged Sarcoma I tumor, indicating that the modified tumors did not lose their capability for growth in A strain mice.

Homograft to BALB/c Mice

Sa I/Wald 2 (donor tumor from A strain mice treated with normal C57BL/6 tissue). The tumor was palpable in all 19 BALB/c mice within 7 days after implantation and achieved a size of 1 by 1 to 1.8

by 1 cm. by the 14th day in each. Five were sacrificed and the tumor transplanted to BALB/c mice for a second passage. In the remaining 14 mice of passage No. 1, the tumor regressed within 60 days. Passage No. 2, in 16 normal adult BALB/c mice yielded a progressive tumor growth in 13 of 16, achieving the same growth size as in passage No. 1. The graft regressed in 10 of these 13 mice; the other 3 were used for transplant to mice for passage No. 3. In this passage, the tumor grew in only 4 of 8 mice, and in the fourth passage, did not survive in any of the 10 mice receiving implants.

Sa I/Wald 3 (donor tumor from A strain mice treated with tumor EO771 tissue). The tumor graft grew in 13 of 18 mice, achieving a size of 1 cm. within 14 days. Three mice were sacrificed for transplant, and the tumor regressed in the remaining 10 mice. In passages No. 2 and No. 3, the tumor grew in 6 of 10 mice respectively, but then regressed in all. In the fourth passage, it did not become palpable in any of the 10 BALB/c mice in which it had been implanted.

Homograft to C57BL/6 Mice

Sa I/Wald 2 (donor tumor from A strain mice treated with normal C57BL/6 tissue). The tumor was palpable in all 20 C57BL/6 mice within 7 days after implantation. It achieved a size of 1.5 by 1.7 cm. in 12 to 15 days and then regressed in all. In the second passage, the tumor implant did not survive in any of 10 mice.

Sa I/Wald 3 (donor tumor from A strain mice treated with tumor EO771 tissue). The tumor graft was palpable in 15 of 20 mice in 7 to 10 days but proceeded to regress in all, never achieving a size greater than 0.5 cm. The progressive rejection of the homografts in the C57BL/6 animals was somewhat surprising in view of the fact that the A strain mice had been treated with C57BL/6 tissues, and, as shown below, the homografts in C3H mice, using identical material, resulted in consistently positive acceptance.

Homograft to C3H Mice

Because of a shortage of C3H mice, passages 3 and 4 of Sa I/Wald 2 and Sa I/Wald 3 were implanted in C3H x BALB/c F-1 hybrids as well as in C3H. The tumors grew in the F-1 hybrids in the same manner as in the C3H strain mice.

Sa I/Wald 2 (donor tumor from A/Jax treated with normal C57BL/6 tissue). In this strain the tumor survived in progressively larger numbers of mice with each succeeding transplantation and is at present in the 22nd passage. With each succeeding passage, the tumor

persisted for longer periods of time. Moreover, it has been observed that while in the first 4 passages the tumor was palpable in 7 to 9 days, in passage No. 5 and thereafter, it was palpable in 3 to 5 days. At the 22nd passage, the tumor grew progressively in 80 per cent of the mice, the latter dying with very large tumors.

This acceptance of the homograft by the C3H mice was entirely consistent with our previous results in adapting the recipient host rather than the donor.[15] It had been observed that when C57BL/6 mice were treated with normal lyophilized A strain mouse tissues, subsequent implantation of Sarcoma I from such mice resulted in greater acceptance by the unrelated C3H strain than by other C57BL/6 mice; this indicated further adaptation of the tumor to its new host strain. This is a phenomenon which requires additional investigation in order to understand its mechanism.

Sa I/Wald 3 (donor tumor from A/Jax treated with tumor EO771 tissue). In the first and second serial passages with implantation directly into normal adult C3H mice, the graft was palpable in 70 to 80 per cent of 20 mice within 7 to 9 days and was transplanted when 1.5 cm. in greatest diameter. The tumor regressed in the surviving mice not sacrificed for tumor transplant.

In the third to ninth passages, the graft was palpable in all 15 mice in each passage in 3 to 5 days; 4 mice were used for transplant purposes, and 80 to 90 per cent of the remaining 11 died with large tumors. At the time of this report, the graft appears to be established in C3H mice with 100 per cent mortality; it is in the 42nd serial passage in this strain and is transplanted in 6 days. This tumor, from the 35th passage in C3H mice, was transplanted to normal DBA/1 mice; in these the tumor (designated Sa I/Wald 4) is in the eighth passage with 100 per cent mortality.

Control Graft to A Strain Mice

Both Sa I/Wald 2 and Sa I/Wald 3, when transplanted to normal A/Jax mice, grew in the usual manner of the unchanged Sarcoma I in A strain mice. Thus, passage through the treated A strain mice did not in any grossly observable way affect the growth pattern of these tumors.

At the 24th serial transplant passage, the altered Sa I/Wald 3 in C3H adult mice was transplanted from the C3H mice directly to 4 mice each of A, BALB/c and C57BL/6 strains. The Sa I/Wald 3 implants survived and grew progressively only in the A strain mice. These died with large tumors. The implant grew temporarily and regressed in the BALB/c strain and did not grow in the C57BL/6 mice.

Histologic Observations

The histologic examination of the tumors in the entire series of animals may be summarized very succinctly. From the overall standpoint there were no significant morphologic variations in the tumors in the animals in which the homograft was accepted. There were marked individual variations in the general architecture of the tumors even within a given passage, but there were no alterations in the 25th passage which could not be found in the first or second passage when multiple sections were examined.

There appears to be little purpose in a detailed histologic description of Sarcoma I as it appears in its native A strain host. Suffice it to say that the tumor was pleomorphic—a mixture of spindle and polyhedral cells, arranged for the most part in bundles having no definite pattern. There was a tendency for the tumor to exhibit perivascular cuffing, especially in the more rapidly growing lesions, where necrosis was a prominent feature. The tumor was highly malignant, infiltrating the dermis and musculature extensively, but it rarely, if ever, metastasized. Varying degrees of anaplasia were found in tumors in individual animals, and even in different parts of the tumor in the same animal.

Certain recognizable trends were observed in the course of examining these multiple transplants, but they were only to be considered in the broadest terms. In the first or second transplant to a normally resistant host, the tumor tended to be more compact and to infiltrate the surrounding tissues in a somewhat more restrained fashion. The number of mitotic figures seemed to be fewer, and necrosis was a relatively minor feature. However, with subsequent passages, the tumor regained its typical architectural pleomorphism with larger numbers of spindle cells, abnormal and more numerous mitotic figures, greater invasiveness, and more necrosis. These comments hold true in both series of animals—those with Sa I/Wald 2 in serial passage and those in which Sa I/Wald 3 was transplanted in serial passage.

Unfortunately, near the latter part of this investigation, an endemic infection in our mouse colony wiped out certain groups of animals and required the termination of the experiment. However, no differences in the structure of the tumors, even in certain secondarily infected animals, were observed.

Summary

Pretreatment of A strain mice with lyophilized C57BL/6 normal or EO771 adenocarcinoma tissues resulted in an alteration in the growth characteristics and strain specificity of subsequently implanted Sar-

coma I. The data indicated that it was possible to alter an adult donor mouse so that subsequently transplanted indigenous tumor underwent adaptive changes which enabled it to grow progressively in an untreated recipient adult host of another strain.

Two altered sublines of Sarcoma I in normal adult C3H mice are described. Sa I/Wald 2 was derived from Sarcoma I implanted in A strain mice pretreated with lyophilized C57BL/6 normal tissue. Sa I/Wald 3 was derived from Sarcoma I implanted in A strain mice pretreated with lyophilized EO771 tumor tissue from C57BL/6 mice.

Direct homotransplantation of altered Sarcoma I (Sa I/Wald 2 and Sa I/Wald 3) to normal adult mice of the C3H strain resulted in a progressively increasing number of mice with tumors which persisted in subsequent passages. Sa I/Wald 3 is presently in the 42nd passage in normal adult C3H mice.

Direct homotransplantation to normal adult mice of the C57BL/6 and BALB/c strains resulted in a temporary growth of the host-altered Sarcoma I in both of these strains. In succeeding passages the implant failed to persist.

The morphologic characteristics of the two altered strains of Sarcoma I (Sa I/Wald 2 and Sa I/Wald 3) did not differ significantly from the original Sarcoma I even after 28 passages in C3H mice.

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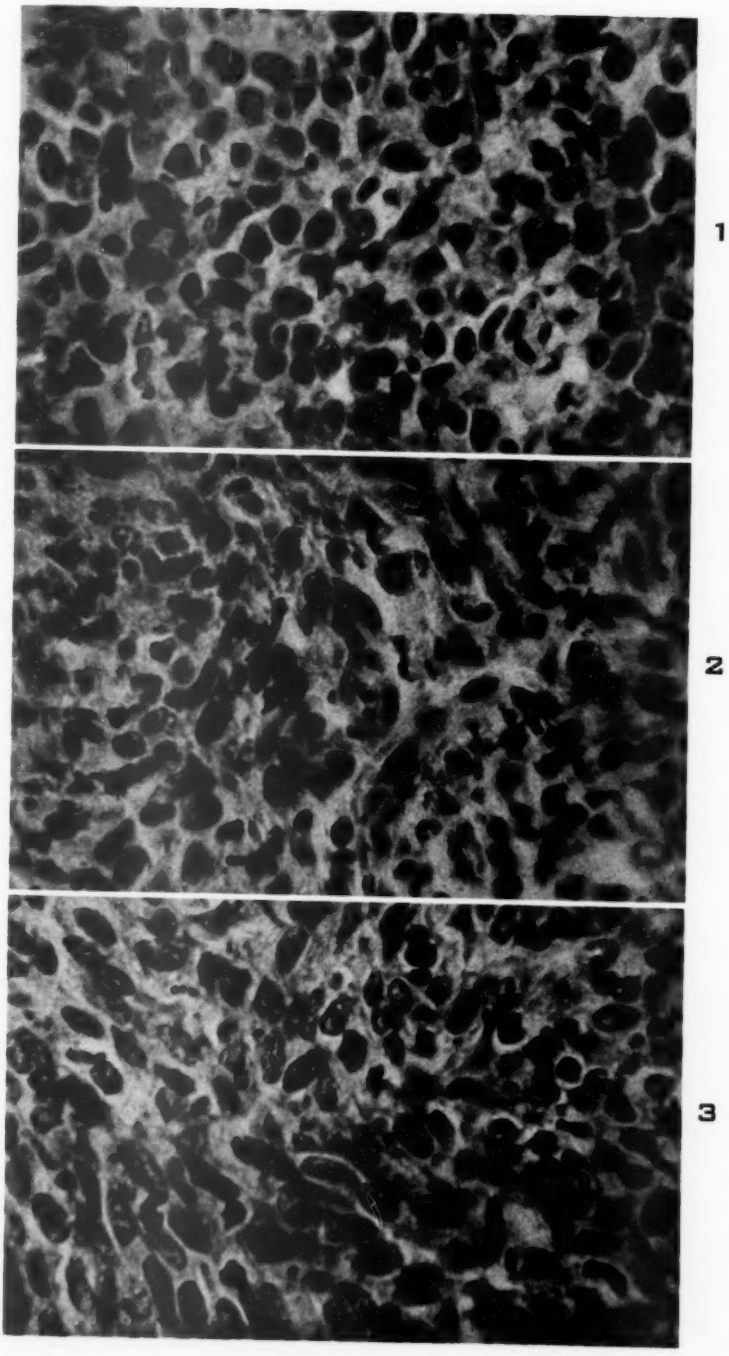
LEGENDS FOR FIGURES

FIG. 1. Sarcoma I in A strain mouse (native host) pretreated with lyophilized tumor Eo771 tissue. Hematoxylin and eosin stain. × 840.

FIG. 2. Sarcoma I homograft in normal adult C3H mouse, 24th passage; tumor origin from treated A strain donor. Hematoxylin and eosin stain. × 840.

FIG. 3. Sarcoma I homograft in normal adult BALB/c mouse, third passage; tumor origin from treated A strain donor. Hematoxylin and eosin stain. × 840.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THE BEHAVIOR OF BASEMENT MEMBRANES IN INTRADUCTAL CARCINOMA OF THE BREAST*

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Soon after Broders introduced the concept of carcinoma *in situ*[1] into the medical literature, it became apparent that it was often extremely difficult to decide whether a neoplasm was confined within the biologic boundaries of the epithelium from which it originated, or had already progressed beyond them and had become invasive. This is common in carcinoma of the female mammary gland, in which the intraductal type of growth frequently shows the cytologic features of malignancy without exhibiting any definite morphologic evidence of invasiveness even when serial sections of the entire lesion have been examined. In other cases, the diagnostic problem is complicated by the innocent appearance of intraductal epithelium which has grown to such an extent as to suggest the possibility of malignancy. Indeed, in the Laboratory of Surgical Pathology, Columbia Presbyterian Medical Center, there have been examples of intraductal carcinoma with axillary lymph node metastases or death from distant spread of neoplasm although careful examination of the mastectomy specimens showed no recognizable evidence of stromal invasion by the carcinoma.

In a previous study[2] it was observed that recognizable basement membranes could be demonstrated in association with intraductal carcinoma of the breast but not in the invasive portions of the same tumor. It seemed of interest, therefore, to investigate in detail the behavior of the basement membranes in examples of intraductal carcinoma of the breast in which no evidence of invasion could be detected by conventional histologic stains. In these cases there was satisfactory follow-up data for at least 5 years post-operatively or to the time of death.

MATERIAL AND METHODS

In the files of the Laboratory of Surgical Pathology, Columbia Presbyterian Medical Center, under the heading of intraductal carcinoma of the breast as determined by criteria outlined by Dr. A. P. Stout, are included those cases in which the neoplasm appears to be confined within the ducts for at least 50 per cent of its extent. This type of neoplasm may be found associated with Paget's disease of the nipple.

* Received for publication, December 4, 1958.

Sections from 180 cases of intraductal mammary carcinoma observed during the period from 1913 to 1952 were examined. Of these, 81 were associated with Paget's disease of the nipple. After reviewing the sections prepared by conventional methods, it was found that 22 cases of intraductal carcinoma, 11 of which were associated with Paget's disease, showed no recognizable evidence of invasiveness. The present investigation was carried out on these 22 cases. In 18 of them (7 were associated with Paget's disease), the original paraffin blocks were available for study. The number of blocks in which neoplastic elements were present varied from 2 to 9 in each case, depending on the size of the lesions. The tissues had been fixed in 10 per cent neutral formalin or Zenker's or Bouin's solutions.

From the paraffin blocks, serial sections were cut and attached to the slides with distilled water. The sections were stained with hematoxylin and eosin and by the Ritter-Oleson method. The latter consists of Hale's procedure followed by staining with the periodic acid-Schiff (PAS) reaction (alcoholic solution of periodic acid).[3] This method was originally intended for the staining of acid and neutral mucopolysaccharides respectively.[3] The method, when used alone, lacks specificity for the exact histochemical determination of acid mucopolysaccharides,[4,5] but it has the advantage of demonstrating the basement membranes of the mammary gland particularly well.[2] Its applicability here is furthermore justified since this investigation is not primarily concerned with histochemical evaluation of acid mucopolysaccharides. With this staining technique, the normal basement membranes are bright purple-red, while the surrounding stroma is stained predominantly blue and in part red. The basement membranes, therefore, stand out clearly. The nuclei stain blue very lightly.

Results

The follow-up data are grouped in Table I from which it can be seen that 19 patients were operated upon by radical mastectomy and 3 by simple mastectomy. Among the former, 16 were living and free of neoplasm from 5 years and 5 months to 23 years and 4 months after mastectomy. Two had axillary metastases at the time of operation and were living, free of disease, 6 years and 3 months, and 10 years after operation. One was found free of lymph node metastasis at the time of operation, but later developed distant metastases which caused death 4 years after radical mastectomy. The 3 patients treated by simple mastectomy alone had no recurrences or evidence of metastasis, and were living from 11 years and 3 months to 13 years following operation.

Histologically, the tumors had the characteristic features of intraductal carcinoma. The neoplastic cells, showing varying degrees of differentiation, filled the ducts and expanded them into large cords. In some cases the patterns of noninfiltrating papillary or comedo carcinoma were present. From the ducts, the tumor was frequently seen to

TABLE I
Intraductal Carcinoma of the Breast

Lesion and treatment	No. of cases	Recurrence	Metastasis: Regional at operation	Metastasis: Distant after operation	Follow-up*
Intraductal carcinoma:					
Radical mastectomy	10	—		—	CC 7 to 23.3
			1		CC 10
Simple mastectomy	1	—	—	—	CC 11.2
Intraductal carcinoma with Paget's disease:					
Radical mastectomy	9	—			CC 5.5 to 20
			1		CC 6.2
				1	DofD 4
Simple mastectomy	2	—	—	—	CC 12.6 to 13

* CC = Clinical cure.
DofD = Died of disease.
The numbers express the interval in years after mastectomy.

extend in intra-epithelial manner into the alveoli. In the cases in which the lesions were associated with Paget's disease, characteristic Paget's cells were present in the nipple, with intra-epidermal location. In none of the multiple sections stained with hematoxylin and eosin was there evidence of invasion into the surrounding stroma.

In the sections stained by the Ritter-Oleson technique it was found, as expected, that the basement membranes of non-neoplastic ducts and alveoli were stained with the normal red-purple color and were uniformly intact. On the contrary, a great deal of variation was observed in the staining of basement membranes in neoplastic foci. Some were sharply outlined and stained red (Figs. 1 and 2). Others appeared indistinct because of thinning out or fading into the surrounding stroma (Figs. 3 and 4) and occasionally stained blue. In still other regions the membranes were not demonstrable at all (Figs. 5 and 6). All or most of these variations were to be found in every case examined and involved the circumference of the membrane either completely or only in part. Of particular interest was the fact that in all cases except one, foci of carcinoma devoid of basement membranes were demonstrable

in small or large numbers. Only in one case, a 51-year-old patient, living and free of disease 23 years and 4 months after radical mastectomy, were the basement membranes completely free of alteration.

In the absence of basement membranes, collections of neoplastic cells, although exhibiting the general architecture of intraductal growth, were found to be surrounded by non-membranous stroma in the same fashion as nests of invading cells in the instances of infiltrating carcinoma. The tumor cells, thus, were in direct contact with stroma, without any interposed limiting structure.

It was also noted that myo-epithelial cells, although irregularly distributed, were present in some foci of intraductal carcinoma surrounded by basement membranes.

One particular distortion of the basement membrane was observed frequently when neighboring neoplastic ducts became confluent. This was characterized at first by what appeared to be fusion of the basement membranes at the point of contact between two or more carcinomatous ducts (Fig. 2). It was followed by disappearance of the most central portion of the fused basement membranes (Fig. 9) and was accompanied by necrosis of the centrally located neoplastic cells. As the result of this process, a large scalloped focus of carcinoma was surrounded by a basement membrane from which fused residual membranous elements projected toward the center of the lesion. The remnants supported cancer cells which were arranged in multiple layers and mimicked papillary projections (Fig. 10).

No attempt was made to interpret the histochemical reaction of the surrounding stroma because the diversity of fixatives used did not allow comparative histochemical evaluation.

Discussion

The observations cited seem to suggest that the alterations in the structure and histochemical reaction of the basement membranes of intraductal lesions of the breast may in some way be related to the benign or malignant nature of the lesion.

It is known that benign lesions of the breast may be accompanied by minor alterations of the basement membrane, but its integrity is not disturbed.[2] On the contrary, the diffusely infiltrating malignant lesions are regularly lacking in basement membranes.[2] The intraductal carcinomas constitute a distinctive group in which some of the ducts are surrounded by more or less well defined basement membranes, with or without histochemical alterations, whereas in others the membranes are very indistinct or barely recognizable. In still other ducts the basement membranes are absent altogether.

For a long time the concept of carcinomatous invasion has been related to a break-through of the basement membrane by neoplastic cells. This, in effect, can be considered as a corollary to the definition of carcinoma *in situ* which, as Broders stated in 1932,[1] is "a condition in which malignant epithelial cells and their progeny are found in or near positions occupied by their ancestors before the ancestors underwent malignant transformation. At least they have not migrated beyond the juncture of the epithelium and connective tissue of the so-called basement membrane. . . ." The importance of the basement membrane as a boundary to carcinoma *in situ* has been stressed recently by Sirtori.[6] Even more recently, Stout,[7] commenting on the involvement of endocervical glands by intramucosal carcinoma of the cervix, has stated that "such involvement is not to be considered invasive carcinoma unless or until there has been a rupture of the basement membrane, and actual invasion of the substantia propria where there are tissue spaces as well as blood and lymphatic vessels."

There must be, therefore, a point at which a carcinoma which has remained for some unknown length of time as purely intraductal (i.e., intra-epithelial) becomes capable of invasion and of metastatic dissemination. It stands to reason that at least in intraductal carcinoma of the breast this crucial moment might be represented by the disruption or disappearance of the basement membrane. It seems that at the time the basement membrane related to a focus of intraductal carcinoma loses its integrity, the neoplastic cells come into direct contact with the surrounding stroma (as in the case of unequivocally infiltrating carcinoma) and with its tissue spaces, blood and lymphatic vessels as well.

In accordance with the classic definition of His,[8] a basement membrane has been considered to be an enveloping membranous structure made up of reticulin fibers arranged in a close and intimate network. These fibers are argyrophilic and PAS-positive.[9] Although the function of the basement membrane is still not entirely elucidated, in some locations, as in the case of the mammary gland, it exhibits a degree of histochemical independence of the contiguous stromal structures.[2]

In the specimens examined in this investigation, a number of changes could be seen progressing from the characteristic normal histochemical and morphologic appearance of the basement membranes surrounding neoplastic ducts to distortion and ultimately, disappearance. In all but one of the cases studied, a few or most of the basement membranes had lost specific histochemical qualities and no longer exhibited staining in normal manner with the PAS and Hale stains. Structurally, the mem-

branes were found to be thinned out, ill-defined, or absent to varying degrees. These alterations are not observed in benign lesions.

One might then postulate that in intraductal mammary neoplasm, whenever the basement membrane cannot be demonstrated by proper staining techniques, the lesion should be considered malignant and, moreover, as having proceeded beyond the ***in situ*** stage. This would hold even though invasion of the stroma is not demonstrable by conventional histologic methods. It might be argued that the ductal pattern does not necessarily reflect neoplastic alteration of pre-existing mammary ducts alone, but may also result from the production of neoplastic cords simulating ductal structure. In the latter instance, such basement membranes as might appear would have been produced in conjunction with neoplastic elements and would not represent pre-existing ductal membranes. In the course of this investigation these possibilities were considered in each instance. In many sections there were numerous large neoplastic ducts in close proximity to each other. Careful scrutiny of these sections, however, gave the impression that the proximity was the result of a considerable expansion of pre-existing ducts by an increase in the number and size of lining cells which had undergone neoplastic alteration and was not attributable to newly formed ductules. It also appeared that the mode of extension of these tumors, at least in their initial phases, was by intra-epithelial progression along ducts and eventually into acini. In addition, the formation of new neoplastic ducts seemed unlikely since the emergence of newly formed ducts of this type from supposedly parent ducts could not be satisfactorily demonstrated. It was felt, to the contrary, that whenever the intraductal carcinoma extended into the surrounding stroma, it did so in the form of frankly invasive nests of cells as in the invasive portions of these tumors (Fig. 7). These nests of invading cells have never been found to be surrounded by basement membranes (Fig. 8). What seems to escape detection in the examination of tumors of this type by conventional histologic methods is the evidence of transformation of the purely intraductal lesion into its invasive form.

It is not intended here to claim that the basement membrane enveloping the carcinomatous duct is that persisting from the normal parent duct. Indeed, it is well known that basement membranes in general are not biologically stable structures, but undergo a continuous turnover in which parenchymal cells play an important role. This is well demonstrated during embryonal development. Nor is it denied that carcinomas may not be capable of forming their own basement membranes. Sirtori[6] has stated that metastatic carcinoma in lymph

nodes may induce the formation of its own basement membrane, as demonstrable by reticulin stains.

It is re-emphasized that in mammary carcinoma, only intraductal lesions have been surrounded by basement membranes; these are not evident in the invasive portions of the tumors. In this connection, numerous metastatic lesions in lymph nodes and in lymphatics have been examined in both the present group of cases and in others as well. Whether well or poorly differentiated, no PAS-staining basement membranes were noted in relation to any of these. In one lymph node metastasis (a well differentiated carcinoma of papillary type), there was a peculiar stromal reaction characterized by a loose network of PAS-positive reticulin fibers which seemed to envelop groups of neoplastic cells in a manner similar to that of basement membranes. However, closer examination disclosed that this was more apparent than real, inasmuch as the fibrils were seen running along the interstices between groups of cancer cells, extending from one group to the next without forming independent enveloping structures. In addition, at the very periphery of the lesion there was no PAS-positive reticulin framework interposed between the neoplastic nodule and the lymph node parenchyma.

Although some carcinomas may induce the formation of basement membranes, this seems to be the exception rather than the rule in neoplasms of the breast. It is possible that in intraductal carcinoma, the neoplastic cells may contribute to the "turnover" of ductal basement membranes as long as their degree of differentiation and slow rate of growth permit them to do so. In the more anaplastic lesions, the ability to contribute to the preservation of the basement membranes appears to be lost. In this case, the absence of basement membranes would not thus be the result of destruction but of a failure of formation. This might be an explanation for the absence of basement membranes in frankly invasive carcinoma and their occasional appearance in the better differentiated neoplasms. In respect to embryonic development Bairati and Toni[10] have stated that the degree of differentiation of the parenchyma is directly related to the degree of differentiation of the stroma (of which the basement membrane is a very significant part).

It follows reasonably, therefore, that the absence of a basement membrane in relation to an intraductal breast tumor may be taken to indicate that the neoplasm is malignant and verges upon invasion. The dividing line between noninfiltrating and infiltrating intraductal mammary carcinoma is reflected by the presence or absence of basement

membranes. Carcinoma *in situ* cannot be satisfactorily demonstrated by conventional histologic methods since these do not demonstrate the behavior of the basement membranes adequately.

The observations cited would, therefore, appear to justify the practice of radical surgical resection in the case of intraductal carcinoma of the breast. The long term clinical survivals in the cases reported, although including those treated by simple mastectomy, only show a more favorable prognosis in this type of neoplasm but do not justify conservative treatment. The two patients with axillary metastases at the time of operation and the patient whose neoplasm became disseminated and who died 4 years after radical mastectomy lend strong support to this point of view.

Summary

The tissues from 180 cases of intraductal carcinoma of the breast have been reviewed. In 22 (11 of which were associated with Paget's disease of the nipple) no evidence of stromal invasion was detected in multiple sections stained by conventional methods. However, two of these patients had regional lymph node metastases and one other died of generalized metastatic neoplasm 4 years after radical mastectomy.

In 18 of the 22 cases, histochemical investigation of ductal basement membranes was undertaken. Focal disruption or complete absence of the membranes or histochemical variations were noted in all cases except one in which the alterations were equivocal. The absence of basement membranes in intraductal carcinoma was considered to represent the first detectable evidence of invasion.

It is felt that intraductal carcinoma *in situ* of the breast cannot be satisfactorily demonstrated by conventional histologic techniques.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Intraductal carcinoma. No evidence of invasion can be detected. Hematoxylin and eosin stain. × 280.

FIG. 2. The same lesions shown in Figure 1 are here stained by the Ritter-Oleson method. Basement membranes are well demonstrated and intact. In some regions they appear to be fusing with each other. × 280.

FIG. 3. Intraductal carcinoma with no demonstrable invasion shown by hematoxylin and eosin staining. × 280.

FIG. 4. The same lesion shown in Figure 3. The Ritter-Oleson stain shows the basement membrane to be thinned and less well defined than in Figure 2. It appears to be fading into the surrounding stroma. × 280.

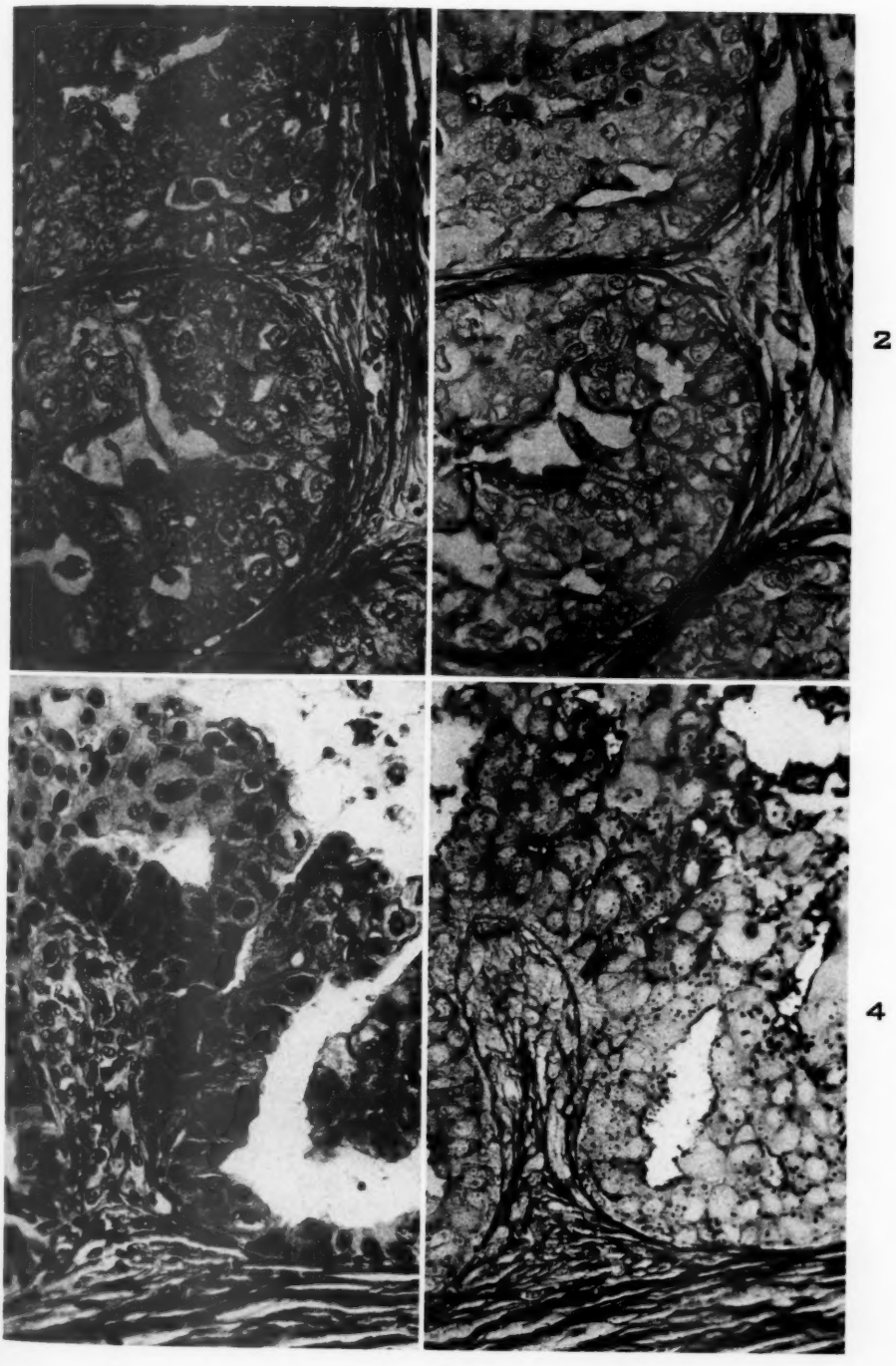
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FIG. 5. Intraductal carcinoma showing no definite evidence of invasion when stained by hematoxylin and eosin. × 280.

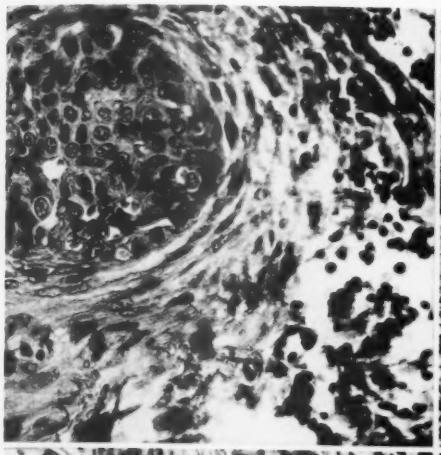
FIG. 6. The same lesion shown in Figure 5. No basement membrane can be demonstrated with the Ritter-Oleson stain. × 280.

FIG. 7. An area of invasion in a mammary carcinoma which otherwise features a predominantly intraductal architecture. Hematoxylin and eosin stain. × 280.

FIG. 8. The same lesion shown in Figure 7. No basement membranes are evident around nests of infiltrating neoplastic cells. Ritter-Oleson stain. × 280.

FIG. 9. Disappearance of the innermost portions of fused basement membranes of adjacent foci of intraductal carcinoma. Ritter-Oleson stain. × 280.

FIG. 10. A more advanced stage than that shown in Figure 9. Pseudopapillation is manifest. Ritter-Oleson stain. × 280.



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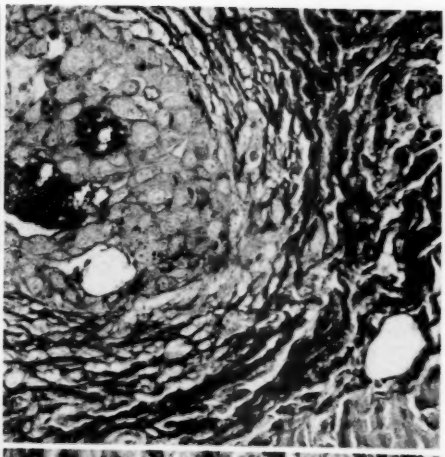

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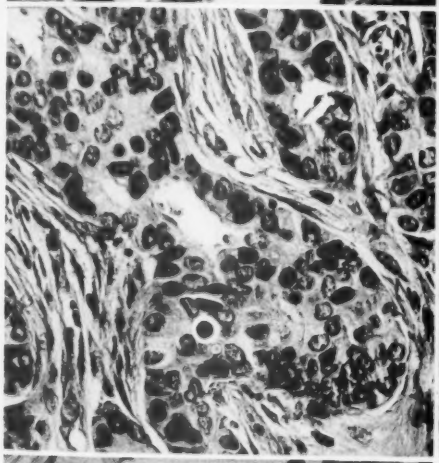

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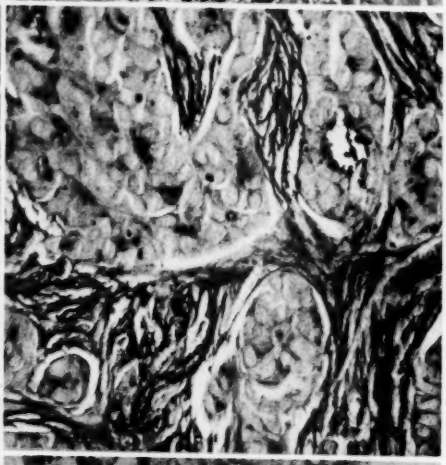

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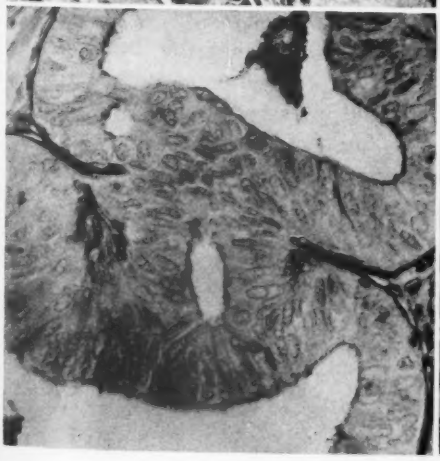

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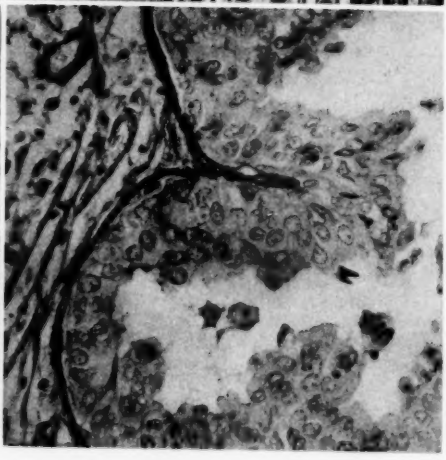

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PHAGOCYTOSIS OF YEAST CELLS *IN VITRO**

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Recently, during the examination of a "lupus cell" (LE) smear,† several intracellular yeast-like elements were observed within monocytic cells in a circumscribed portion of the preparation. At first glance, the intracellular yeast cells were mistaken for *Histoplasma capsulatum*. Thorough investigation of the two persons whose blood had been used in making the preparation yielded no evidence to indicate that either one had active histoplasmosis or logically could be considered a carrier of the organism in his peripheral blood. In view of this and the observations to be reported, it would appear that the intracellular yeast cells represented saprophytic organisms.

Histoplasma capsulatum is a yeast-like organism with slightly oval shape which measures 1 to 5 μ. It can be demonstrated occasionally in mononuclear cells and neutrophils in peripheral blood or bone marrow smears.[2] This is considered to be a highly suggestive observation and is accepted by many as a diagnostic feature of histoplasmosis. Most authorities agree, however, that successful cultural isolation of the Histoplasma is a preferable method of establishing the diagnosis.[2]

Since the chance occurrence cited above may mislead the casual observer, it is considered desirable to describe the general features of phagocytosis of yeast-like organisms *in vitro*.

Material and Methods

The organisms used were *Hansenula anomala, Saccharomyces carlsbergensis* (ATCC 9080), *Candida albicans* No. 1, *Candida albicans* No. 2, *Cryptococcus neoformans* (ATCC 10226), *Cryptococcus neoformans* (pigeon nest strain 48), *Cryptococcus neoformans*, variety innocuous, *Histoplasma capsulatum, Histoplasma duboisii Vanbreuseghem, Blastomyces dermatitidis*, and *Blastomyces brasiliensis*. *Histoplasma capsulatum, Blastomyces dermatitidis* and *Blastomyces brasiliensis* were grown in the yeast phase on Kurung's egg medium at 37° C.[3] All other organisms were cultured on Sabouraud's dextrose broth and kept at room temperature.

For direct observation of phagocytosis, oxalated blood was kept

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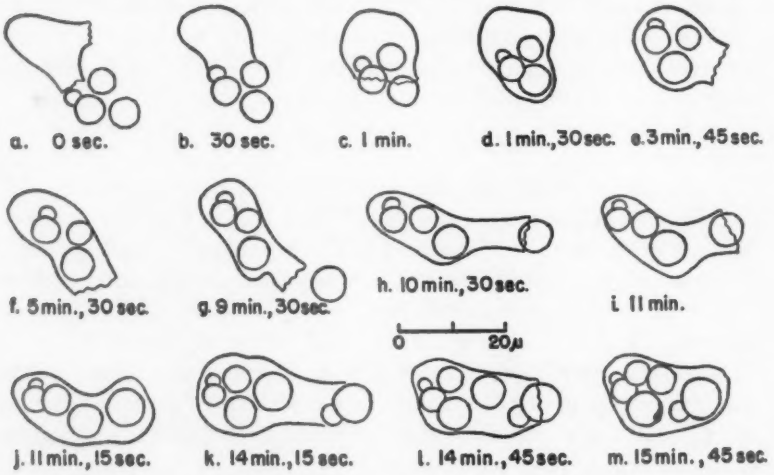
† The smear was prepared according to the "Snapper ring technique" for detecting the "LE factor" in patients with systemic lupus erythematosus.[1]

until the erythrocytes had settled, at which time a few loopfuls of the buffy coat were placed on a clean slide. A loopful of one of the cultures listed above was mixed with the suspension of leukocytes on the slide. A cover slip, elevated by applying Vaseline® around its edges, was then placed over each mixture, and the preparation was examined microscopically.

For permanent smears, the best preparations were obtained by mixing either the oxalated whole blood or the white cell layer with a loopful of one of the cultures, allowing 30 minutes for "incubation" at room temperature. A loopful of the leukocyte-yeast mixture was then spread on the slide and stained with Wright's stain.

Results

In wet preparations, phagocytosis was readily observed after the culture and white cells were mixed. Pseudopods projected from neutrophils, one or more at a time, and the cell moved in an ameboid fashion. As soon as the pseudopod came into contact with a yeast cell, the organism was engulfed by the flowing cytoplasm. Usually this process took 30 to 60 seconds. Examples of phagocytosis of yeast



Text-figure 1. *In vitro* phagocytosis of *C. albicans* No. 1. Drawn from wet preparation with the aid of the camera lucida.

forms are shown in Text-figure 1, in which various stages of ingestion of *Candida albicans* by a leukocyte are illustrated. Within one minute the leukocyte engulfed a cluster of 4 yeast cells simultaneously. (Text-figure 1, a to d.) Thereafter, pseudopods extended toward a nearby

yeast cell which was in turn engulfed a minute later (Text-figure 1, e to j). Figures a, k, l, m (Text-fig. 1) show the leukocyte ingesting a budding yeast form within a 90-second period. Thus, 7 yeast cells were completely engulfed in 15 minutes and 45 seconds.

A single leukocyte often ingested as many as 10 yeast cells in short periods of time if the concentration was a heavy one. This was the case with all the organisms tested. Occasionally the leukocyte came into contact with one portion of a pseudomycelium and moved along it in one direction.

Phagocytosis was observed in freshly drawn blood and in blood which had been kept for 3 to 5 hours at room temperature. Leukocytes in some samples appeared to be more active than in others. Neutrophils, monocytes, as well as eosinophils, demonstrated this property.

The yeast-like organisms found in the original LE preparation are shown in Figure 1. These cells were oval and measured from 1.5 to 2.8 by 2.8 to 5.5 μ. In some, darkly staining, crescent-shaped chromatin substance was encountered at one end; others were poorly stained and appeared devoid of chromatin. Many contained pink-staining cytoplasm with or without a dark-staining spot. Table I summarizes the histologic features of the organisms in their intracellular location.

TABLE I

Phagocytosis of Fungi: Intracellular Appearance

Organism	Appearance	Measurements (μ)
Hansenula anomala	Oval, oblong, cylindrical	3.6×2 (3 to 5.5) × (2 to 3.6)
Saccharomyces carlsbergensis	Oval, oblong, cylindrical	5.4×3.6 (3.6 to 7.2) × (2.7 to 4)
Candida albicans No. 1	Oval, spherical	4.5×3.6 (2.7 to 7) × (2 to 3.6)
Candida albicans No. 2	Oval, spherical	3.6×2.7 (2.7 to 4.5) × (1.8 to 3.6)
Cryptococcus neoformans No. 48	Spherical, oblong	5 (3.6 to 6) in diameter
Histoplasma capsulatum	Oval, oblong	3.5×2.8 (2.8 to 4.5) × (2 to 3)
Histoplasma duboisii	Oval, oblong, spherical	4×3.6 (3.4 to 6.4) × (2 to 3.6) 2 to 6.5 in diameter
Blastomyces dermatitidis	Spherical, oblong	6.4 to 9 in diameter
Blastomyces brasiliensis	Oblong, oval, spherical	(3 to 9) × (2 to 7)

Figures 2 to 12 illustrate some of the phenomena observed *in vitro*. The appearance of phagocytized *H. capsulatum* was similar to that observed in blood smears of patients with clinical histoplasmosis. The cells were oval in configuration and contained densely stained chromatin at one end. Most of the engulfed cells measured 2.8 by 3.5 μ (Table I).

The structural characteristics of most of the intracellular forms of *Hansenula anomala* (Fig. 3), *Candida albicans* (Fig. 6), small *Saccharomyces carlsbergensis* (Fig. 4), *Histoplasma duboisii* (Fig. 7), and *Blastomyces brasiliensis* (Fig. 8) were very similar to those of *Histoplasma capsulatum* (Fig. 2). Large oblong and cylindrical forms of Hansenula and Saccharomyces (Fig. 5) were, however, readily distinguishable. *Histoplasma duboisii* was often characterized by a mixture of small and large spherical cells, a feature which served to differentiate this organism from *H. capsulatum*. Although the small forms of *B. brasiliensis* (Fig. 8) resembled Histoplasma, there were often larger forms (9 μ; Fig. 9).

Cryptococcus neoformans in the phagocytized state was usually spherical but occasionally assumed an oblong shape. Some of the organisms were darkly stained, or contained dense chromatin substance at one end (Fig. 10); most of them, however, were faintly stained and were characterized by broad capsules (Fig. 11). *Cryptococcus neoformans*, var. innocuous (Fig. 11) resembled *C. neoformans* in all respects. *Blastomyces dermatitidis* ordinarily exhibited characteristic features. The organism was large, measuring 6.4 to 9 μ in diameter, and was possessed of a darkly stained cell wall (Fig. 12). The cell membranes of the large forms of *B. brasiliensis* were thinner and occasionally exhibited multiple bud production (Fig. 9).

Discussion

Our observations indicate that the phagocytosis of yeast cells by leukocytes can be demonstrated readily *in vitro*. An outstanding feature, however, is the resemblance of many saprophytic yeast cells to *Histoplasma capsulatum*. This possible source of error certainly warrants consideration when blood smears or bone marrow or buffy coat preparations are used in efforts to establish the diagnosis of histoplasmosis. The requisites of sterile instruments, clean slides, and carefully washed skin are obligatory. In the instance provoking this investigation, in which yeast cells were observed within leukocytes in an "LE smear," it was apparent that the spores were contaminants on the slide at the time blood was deposited. In one preparation these organisms were indistinguishable from Hansenula, Saccharomyces or Candida. The size, contour, and staining properties of the yeast cells encountered under these circumstances should be carefully evaluated before a diagnosis of histoplasmosis is rendered on morphologic features alone. Of some interest is the narrow clear space or "halo" surrounding many of the organisms when in intracellular location. Milne[4] investigated the structure and cytochemistry of *H. capsulatum* and

concluded that this was not truly an indication of encapsulation. He observed that the "halo" appeared only in those instances in which alcohol was applied for purposes of staining or dehydration. In the present studies, Wright's stain was used; this, of course, is a methyl alcohol solution.

Conclusions

1. In the course of examining an "LE smear," yeast cells resembling *Histoplasma capsulatum* were encountered within leukocytes.

2. Phagocytosis of yeast cells by leukocytes was shown to occur in oxalated blood *in vitro*.

3. The structural characteristics of phagocytized *H. anomala, C. albicans, S. carlsbergenis, H. duboisii,* and *B. brasiliensis* were similar to those of *H. capsulatum*.

4. Under the conditions utilized, *H. anomala, S. carlsbergensis, C. neoformans, C. neoformans,* var. innocuous, *B. dermatitidis, B. brasiliensis,* though readily engulfed by leukocytes, could be differentiated from *H. capsulatum* only if a large number of leukocytes containing engulfed organisms were examined. It was difficult to distinguish *H. anomala* from *S. carlsbergensis,* and *C. neoformans* from *C. neoformans,* var. innocuous.

5. Cleanliness and even sterility of instruments and glassware is an essential to the preparation of blood or marrow preparations to be used for the diagnosis of histoplasmosis.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIGS. 1 to 12. Various phagocytized organisms in leukocytes. All preparations are stained with Wright's stain. × 1,400.

FIG. 1. "LE smear," showing intracellular yeast-like organism.

FIG. 2. *Histoplasma capsulatum.*

FIG. 3. *Hansenula anomala.*

FIGS. 4 and 5. *Saccharomyces carlsbergensis.*

FIG. 6. *Candida albicans* No. 1.

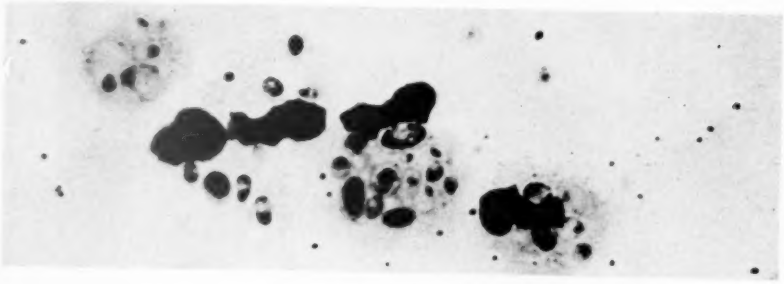
FIG. 7. *Histoplasma duboisii.*

FIGS. 8 and 9. *Blastomyces brasiliensis.*

FIG. 10. *Cryptococcus neoformans.*

FIG. 11. *Cryptococcus neoformans,* var. innocuous.

FIG. 12. *Blastomyces dermatitidis.*



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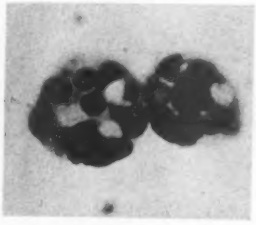

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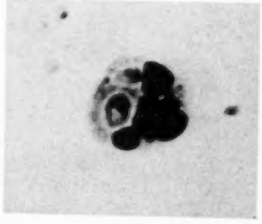

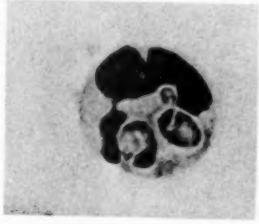

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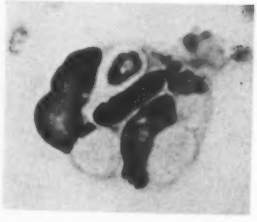

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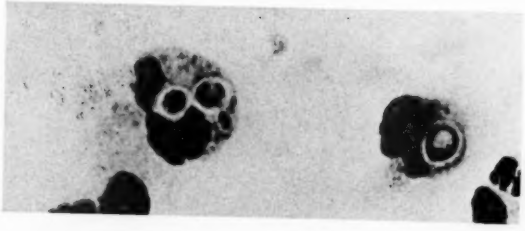

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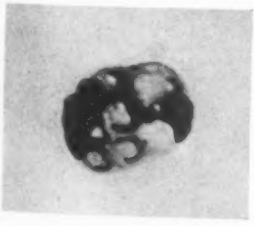

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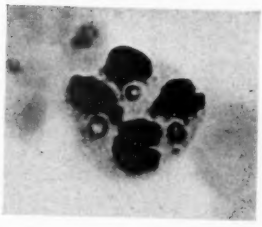

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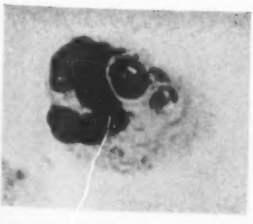

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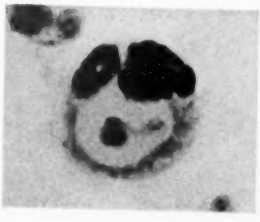

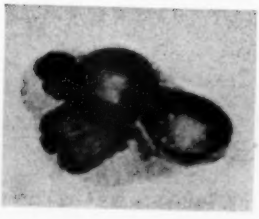

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